

# Dietary cholesterol absorption, and sterol and bile acid excretion in hypercholesterolemia-resistant white rabbits

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**Abstract** The New Zealand white (NZW) rabbit fed a 0.1% cholesterol-enriched diet (CD) typically responds (normoresponsive, NR) by quickly developing hypercholesterolemia. To study the underlying mechanisms responsible for the widespread phenomenon of inter-individual variability of response to dietary cholesterol, a unique hypercholesterolemia-resistant (RT) rabbit model was developed. These animals were utilized to investigate selected potential mechanisms that might enable the RT animal to compensate for dietary cholesterol overload. When rabbits were fed the low-cholesterol stock diet, there was no significant difference in the plasma cholesterol concentrations of the NR and the RT animals. However, a significant rise was observed in the NR rabbits within 1 month of their being placed on the cholesterol-enriched diet; the plasma cholesterol concentration of the RT animals was not affected. During consumption of the cholesterol diet the cholesterol absorption rate was somewhat greater in the NR rabbits ( $P < 0.05$ ), whereas intestinal transit times and the fecal excretion of neutral steroids were substantially the same in both groups. In contrast, the fecal bile acid excretion of the RT animals was more than twice as great ( $P < 0.0001$ ) as that of the NR animals. ■ We conclude that the response to dietary cholesterol is a heritable trait in these rabbits and that, although less dietary cholesterol was absorbed by the RT animals, it appears that a major mechanism controlling plasma cholesterol levels involves the rate of conversion of cholesterol to bile acids and their subsequent excretion. —Overturf, M. L., S. A. Smith, A. M. Gotto, Jr., J. D. Morrisett, T. Tewson, J. Poorman, and D. S. Loose-Mitchell. Dietary cholesterol absorption, and sterol and bile acid excretion in hypercholesterolemia-resistant white rabbits. *J. Lipid Res.* 1990. 31: 2019–2027.

**Supplementary key words** cholesterol diet • plasma cholesterol • fecal excretion • lithocholic acid • deoxycholic acid

The typical New Zealand white rabbit rapidly responds to a cholesterol-enriched diet by developing hypercholesterolemia. This response is characterized by increased LDL and  $\beta$ -VLDL-cholesterol, and a concomitant decrease of HDL-cholesterol. In contrast to the triglyceride-rich VLDL

present in these rabbits when fed a regular, low-cholesterol laboratory chow, the VLDL becomes enriched in cholesteryl esters relative to the triglycerides (1, 2). Increased ingestion of dietary cholesterol commonly has similar effects in other species as well, although the response differs in magnitude. Specifically, a cholesterol-enriched diet typically results in hypercholesterolemia, with the appearance of cholesteryl ester-rich (CER)-VLDL, in rats (3), dogs (4), monkeys (5), and, to a lesser extent, humans (6, 7).

Not only are there large differences in the cholesterol-emic response among different species, there are also large inter-individual responses of plasma cholesterol and lipoprotein concentrations within various species to changes in dietary cholesterol intake. This variability in plasma cholesterol responsiveness within a species was first observed in rabbits (8), and the phenomenon was soon described in several other species including pigeons (9), mice (10), nonhuman primates (11), and humans (12). Although there is substantial evidence that much of this variability in plasma cholesterol response is under genetic control (13–17), the operative mechanism(s) directly responsible have not been defined.

Adams, Gaman and Feigenbaum (18) examined two breeds of rabbits (New Zealand white and Dutch belted) which, when fed a cholesterol supplemented diet, displayed contrasting susceptibilities to the development of hypercholesterolemia. They observed no differences in catabolism or excretion of cholesterol, absorption of cholesterol from the

Abbreviations: VLDL, very low density lipoproteins; CER, cholesteryl ester-rich; LDL, low density lipoprotein; HDL, high density lipoprotein; RT, hypercholesterolemia-resistant rabbit model; NR, nonresponsive; NZW, New Zealand White; CD, cholesterol-enriched diet.

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gut, or cholesterol biosynthesis. On the other hand, recent studies of two inbred strains of JAX rabbits, IIIIVO/JU and AX/JU that are known to be hypo-responsive and hyper-responsive, respectively (19), indicated that differences in the stimulation of bile acid excretion after cholesterol feeding and the efficiency of cholesterol absorption are important determinants of the phenomenon of hypo- and hyperresponsiveness in these rabbits (20).

We have recently developed, by selective breeding, a colony of New Zealand white (NZW) rabbits that is remarkably resistant to the development of hypercholesterolemia (21). For example, when fed a 0.1% (wt/wt) cholesterol-enriched diet for up to 8 weeks, the resistant rabbits (RT) maintained plasma total cholesterol concentrations of < 50 mg/dl, whereas normal rabbits (NR) developed levels of > 300 mg/dl. The constituents of the major lipoprotein classes were also quite different. Whereas CER-VLDL was the major plasma lipoprotein of normal, cholesterol-fed rabbits, HDL was the most abundant lipoprotein of the RT animals. The hydrated density of HDL from NR rabbits was also greater than that of the RT animals, and the LDL of the RT animals contained a lower proportion of esterified cholesterol and protein than the LDL of normal rabbits. LDL binding studies indicated that peripheral mononuclear cells from RT rabbits bound, internalized, and degraded significantly more LDL than did mononuclear cells from normal rabbits (21).

The purpose of the present study was to determine whether differences also exist between the two groups of animals in the rate of intestinal absorption of dietary cholesterol or the excretion of fecal cholesterol and bile acids. Our results indicate that, compared to the NR rabbits, the RT rabbits absorbed somewhat less cholesterol but excreted about the same amount of cholesterol. However, we observed more than a twofold increase in the excretion rate of bile acids by the resistant animals.

## MATERIALS AND METHODS

### Animals

All of the rabbits were of the NZW breed and were derived from progenitors of a large, closed colony. Two major sub-colonies were established from these animals based upon their relative cholesterolemic response to a cholesterol-enriched diet. Animals considered normoresponsive (NR), in that they quickly developed hypercholesterolemia, comprised one phenotypic group. The other group, included rabbits that showed no statistically significant cholesterolemic response to the challenge diet and were therefore considered to be resistant (RT). The derivation of these two colonies, the development of the pedigrees, and the statistically based criteria for the classification of the NR and RT phenotypes have been described (21).

### Housing and diets

To minimize coprophagy all animals were housed in individual metabolic cages with stainless-steel floors comprised of rods spaced 0.5 in apart. The room was temperature-controlled at  $20 \pm 2^\circ\text{C}$  with a 12-h light/dark cycle. The rabbits were fed regular laboratory Purina rabbit chow until they were at least 5 months old. Subsequently, this chow was enriched with cholesterol (0.1% wt/wt) and fed for a minimum of 2 months in order to establish the phenotype of the rabbits with respect to cholesterolemic responsiveness as previously defined (21).

### Reagents and chromatographic materials

The radioactive sterols ( $1\alpha$ ,  $2\alpha$  (n)- $^3\text{H}$ ]cholesterol, and [ $^{14}\text{C}$ ] $\beta$ -sitosterol were purchased from Amersham Corp., Arlington Heights, IL. Radiopurity of both isotopes as determined by thin-layer chromatography in a solvent system of hexane-ethyl acetate 3:1 was >98%. The fatty acid standards, heptadecanoic acid and oleic acid, were obtained from Sigma (St. Louis, MO). The unlabeled sterols were purchased from two sources:  $5\alpha$ -cholestane, cholesterol, sitosterol, 4-cholesten-3-one, and  $5\alpha$ -cholestan-3-one (Sigma); and stigmastanol, campesterol, and coprostanol (Research Plus, Inc., Bayonne, NJ). Bile acids were obtained from several sources: nordeoxycholic acid (Research Plus); deoxycholic acid and lithocholic acid (Aldrich Chemical Co., Milwaukee, WI); cholic acid (Sigma); and lithocholic acid [carboxyl- $^{14}\text{C}$ ] (Amersham Corp.).

4-Dimethylaminopyridine was purchased from Aldrich. All other solvents and reagents were reagent grade and purchased from Fisher Scientific Co., Houston, TX.

### Experimental protocol

Six normoresponsive (NR) and six resistant (RT) age-, sex-, and weight-matched rabbits were used in these studies. All of the animals were fed the 0.1% cholesterol-enriched diet for at least 3 months prior to the studies. The RT animals were in steady state with regard to serum cholesterol levels; the NR were not.

Briefly, to assess the absorption of cholesterol, normoresponsive and resistant rabbits were given a single test meal containing [ $^3\text{H}$ ]cholesterol and [ $^{14}\text{C}$ ]sitosterol. Labeled sterol and bile acid fractions excreted in the feces were serially measured (22-25). Recovery of the largely nonabsorbable  $\beta$ -sitosterol permitted correction for possible losses of cholesterol radioactivity via degradation and an estimate of intestinal transit time (24). The amount of tritium [ $^3\text{H}$ ] in the plasma, derived from the ingested [ $^3\text{H}$ ]cholesterol, was determined concurrently.

After an overnight fast, members of a sex- and weight-matched pair of NR and RT animals were each fed 3 g of rabbit chow, prepared as a 6-g batch. Each batch contained [ $^3\text{H}$ ]cholesterol (6  $\mu\text{Ci}$ ; 47 Ci/mmol) and [ $^{14}\text{C}$ ]sitosterol (0.12  $\mu\text{Ci}$ ; 56 mCi/mmol) added to the chow in a vehicle of chloroform as described for the preparation of a cholesterol-

enriched diet (21). Upon consuming the hand-fed test diet which required only a few minutes, each rabbit was given the remainder (97 g) of the daily ration of cholesterol-enriched chow. This method of administration assured that all of the labeled compounds would be consumed, and permitted reliable assessment of cholesterol absorption as it occurs in the presence of other dietary components during the course of a meal. Twenty-four hours later each rabbit was given the usual daily allotment of 100 g of the cholesterol-supplemented diet (CD), which was typically consumed within a 24-h period. The animals were maintained on the regimen for the next several months.

Total stool and urine specimens were collected over 24-h intervals for the next several days. Plasma samples were taken periodically over the same time period. In addition, some plasma samples were obtained within hours of consuming the test meal. To minimize ear trauma, phlebotomy was not performed on any one rabbit at every time interval.

The stool and urine samples were frozen at the end of each 24-h collection period and dried under vacuum. The dry weight of each fecal sample was determined and the entire sample was ground to a fine state.

Duplicate 0.5-g portions of each powdered fecal sample were used to determine sterol and bile acid content (23, 24). The samples were saponified with 1 N NaOH in 95% ethanol for 1 h at 90 °C. The neutral sterols were then extracted by repetitively (4 ×) partitioning with the addition of hexane (bp 68–70 °C), followed by low speed centrifugation. The pooled upper phases were used to determine labeled sterol content by taking 0.2-ml duplicate aliquots to dryness in a scintillation vial and adding 15 ml of scintillation fluid.

The lower aqueous phases containing conjugated and free bile acids were extracted 4 × with chloroform-methanol 2:1 (v/v), washed once with water, and the lower phases containing the bile acids were pooled and their radioactivity was determined on duplicate samples. Duplicate 0.2-ml aliquots of each sample were dried in scintillation vials prior to the addition of four drops of 2 N HCl and scintillation fluid.

Lyophilized urine specimens were suspended in 200 ml of water and total lipids were extracted with chloroform-methanol 2:1 (v/v). Duplicate samples were placed in scintillation vials, taken to dryness, and counted.

### Radioactivity measurements

The radioactivity of all samples was measured by scintillation counting in 15 ml of Hydrofluor (National Diagnostics, Manville, NJ). After the addition of the scintillation fluid the samples were aged 24 h to eliminate chemiluminescence prior to counting. The disintegration per minute (dpm) per vial was determined by correcting for counting efficiency by the external standard channel ratio method. All samples were counted to a two sigma error of 3%.

Cholesterol absorption was calculated from the formula:

Percent absorption =

$$\left[ 1 - \frac{{}^3\text{H}/{}^{14}\text{C in fecal neutral sterols}}{{}^3\text{H}/{}^{14}\text{C in administered oral dose}} \right] \times 100.$$

where  ${}^3\text{H}$  represents labeled cholesterol and  ${}^{14}\text{C}$  represents labeled sitosterol.

The cumulative fecal  ${}^3\text{H}$  radioactivity in the neutral sterol fraction from day-1 through day-6 was used for the absorption calculation.

### Quantitation of individual fecal neutral sterols and bile acids

Aliquots (100 mg) of powdered fecal samples were used to quantitate the predominant individual fecal sterols and bile acids (26–28). After the addition of 0.25 mg of nor-deoxycholic acid as an internal standard, each sample was saponified in 2.5 M NaOH-methanol. The samples were then taken to pH 1, extracted with chloroform-methanol 2:1 (v/v), and dried prior to derivatization. Butylation of the samples was performed as described (26). The dry butyl ester mixture was then acetylated in a solution of chloroform, dimethylaminopyridine, and acetic anhydride at 40 °C for 16 h. The butyl ester-acetates were extracted with chloroform, back-washed with water, passed through magnesium sulfate-potassium carbonate 1:1 (wt/wt) to remove water, taken to dryness, and dissolved in chloroform prior to gas chromatography (GC).

Routine capillary gas chromatography was done with a Hewlett-Packard 5890 chromatograph fitted with an electron capture detector. A DB-1701 megabore (I.D. = 0.5 mm × 15 m) fused-silica column [polymethyl (50% phenyl) siloxane, 0.25 μm film thickness] obtained from J&W Scientific (Folsom, CA) was used to separate fatty acids, sterols, and bile acids as their butyl ester-acetate derivatives (27). Peak-area correction factors [F(W)] were determined using data obtained from quantitative mixtures of standard compounds (28).

Gas chromatography-electron impact mass spectrometry (GC-MS), using the same column, was used for confirmation of the resolved derivatives. In addition, aliquots of the lyophilized fecal samples were saponified under mild conditions (10% NaOH at 110 °C), separated into neutral and acidic fractions, and extracted with methyl *tert*-butyl ether prior to trimethylsilyl derivatization (TMS). Both derivatives were then subjected to GC-MS analysis.

Twenty-four hours before feeding the radioactive test bolus, stool samples were obtained from NR and RT rabbits and lyophilized. Duplicate aliquots (100 or 500 mg) of the dried fecal specimens were taken from five samples for recovery determinations. Approximately 10,000 dpm of carrier-free, radiolabeled cholesterol, sitosterol, or lithocholic acid was added to each duplicate aliquot; one aliquot was used as a control with no nuclide added. The specimens were saponified and extracted as described (23, 27), assayed for

radioactivity, and the recovery was calculated for each compound.

### Statistics

The results given in the figures are presented as means  $\pm$  standard errors (SEM), and the results given in the table and text are given as standard deviations (SD) of the means. The significance of difference between means was determined with the unpaired Student's *t*-test. The level of significance was chosen to be  $P < 0.05$ .

## RESULTS

A general characterization of the animals at the time they were fed the test meal is given in Table 1. Of the 12 animals studied there were two normoresponsive (NR) and two resistant (RT) age- and weight-matched does and four NR and four RT bucks. The group mean of the plasma cholesterol concentration for the normoresponsive rabbits was  $543 \pm 210$  mg/dl compared to that of the resistant group which was  $49 \pm 14$  mg/dl. The difference was highly significant ( $P < 0.002$ ).

There was no major difference between the NR and the RT rabbits in either daily food consumption or average body weight determined monthly ( $P < 0.05$ ) during the experimental period (Fig. 1). All of the animals appeared healthy and none exhibited excessive hair loss, xanthomatous cutaneous lesions, or fatty deposits in the eyes, which are manifestations commonly observed in rabbits fed larger quantities of cholesterol.

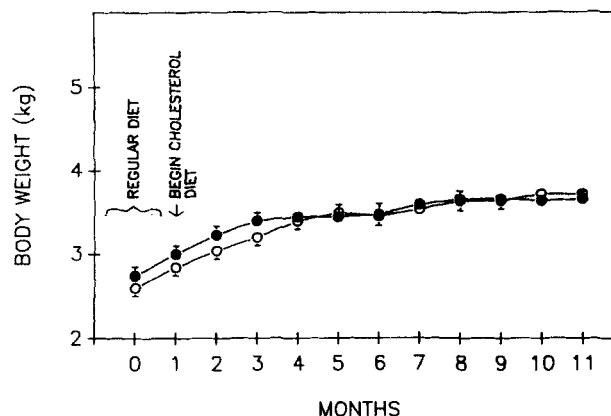


Fig. 1. Growth rate of normoresponsive and hpercholesterolemia-resistant rabbits. The data represent the body weights of the group ( $n = 6$ ) of normoresponsive ( $\circ$ ) and the group ( $n = 6$ ) of cholesterol-resistant rabbits ( $\bullet$ ). The (0.1%) cholesterol-enriched diet was begun at the beginning of month-1. The cholesterol absorption studies were performed during the month-4 through month-6 period. Body weights are given as group means  $\pm$  SEM. There was no significant difference in the values at any time interval between the normoresponsive and the resistant group by the unpaired *t*-test at  $P < 0.05$ .

The RT and NR rabbits had similar plasma cholesterol levels while consuming the regular, low-cholesterol rabbit chow. However, there was an immediate and sharp increase in cholesterolemia exhibited by the normoresponsive rabbits with the feeding of the 0.1% cholesterol-enriched diet (Fig. 2). Over the 16-week period during which time the animals were fed the 0.1% cholesterol diet, the plasma cholesterol level of the NR rabbits increased more than 12-fold

TABLE 1. Characteristics and cholesterol absorption of normoresponsive and resistant rabbits fed a 0.1% cholesterol-enriched diet

Rabbit #	Sex	Wt	Plasma Cholesterol	Cholesterol Absorption <sup>a</sup>
		kg	mg/dl	mg/kg body wt
<b>Normoresponsive</b>				
516	F	3.99	589	16.56
317	F	3.51	458	23.53
601	M	3.54	227	20.59
354	M	3.36	658	23.45
552	M	3.55	479	22.28
361	M	3.09	851	25.59
Mean $\pm$ SD <sup>b</sup>		3.50 $\pm$ 0.29	543 $\pm$ 210	22.00 $\pm$ 3.13
<b>Resistant</b>				
721	F	3.89	59	16.96
719	F	3.54	54	19.83
366	M	3.48	71	17.90
716	M	3.44	36	20.52
717	M	3.62	40	14.44
709	M	3.51	37	20.17
Mean $\pm$ SD <sup>b</sup>		3.58 $\pm$ 0.16	49 $\pm$ 14	18.30 $\pm$ 2.35
<i>P</i> <sup>c</sup>		> 0.005	< 0.002	< 0.05

<sup>a</sup>Sitosterol-corrected values for mass of cholesterol absorbed over 6 days in the isotope-containing test meal.

<sup>b</sup>Group data expressed as the group mean  $\pm$  standard deviation.

<sup>c</sup>*t*-Test of significance between normoresponsive and resistant rabbits.

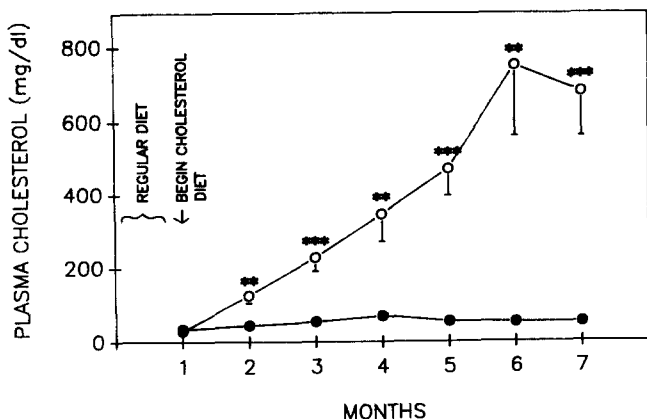


Fig. 2. Plasma cholesterol levels in normoresponsive (○) and hypercholesterolemia-resistant rabbits (●). The cholesterol-enriched diet was begun at month-1. The results are expressed as means  $\pm$  SEM for the six animals per group; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; comparing monthly normoresponsive rabbit values with resistant rabbit values by unpaired  $t$ -test. The absence of a symbol indicates no difference ( $P > 0.05$ ).

from  $38 \pm 3$  to  $470 \pm 70$  mg/dl. The RT rabbits that were fed the same diet over the identical time period showed only a slight increase in mean cholesterol concentrations from  $32 \pm 2$  to  $55 \pm 4$  mg/dl.

The amount of isotope in the total plasma volume was approximated by multiplying the  $^3\text{H}$  radioactivity (disintegrations per minute) in a measured plasma specimen by the estimated plasma volume factor of 35 ml/kg body weight (29). Because there were no significant differences between body weights of the rabbits at the time of the cholesterol absorption studies, small differences in blood volume did not have a major impact on the results.

The radioactivity in the plasma arising from the [ $^3\text{H}$ ]cholesterol test meal reached a peak at 24 h in both groups (Fig. 3). At this time interval there was more than a fivefold increase in the amount of label in the plasma of the NR rabbits compared to the amount in the plasma of the RT animals. This magnitude of difference generally persisted for the 9 days that the plasma radioactivity was measured after the administration of the [ $^3\text{H}$ ]cholesterol bolus.

The results shown in Fig. 3 revealed that considerably more  $^3\text{H}$  radioactivity from the test meal occurred in the plasma of NR rabbits at any one time, and remained there longer. The serum levels of  $^{14}\text{C}$  activity originating from the [ $^{14}\text{C}$ ]sitosterol were below the levels of detection in both groups, indicating that very little was absorbed by the intestine.

The daily volume of urine excreted per rabbit was highly variable and no significant difference was observed between the two groups of animals. Similarly, the amount of  $^3\text{H}$  radioactivity arising from the ingested [ $^3\text{H}$ ]cholesterol was also quite variable, but never totaled more than 5% of the amount of ingested  $^3\text{H}$  radioactivity. The difference between group means was not significant.

Intestinal cholesterol absorption was measured concurrently to determine whether a difference in the rate of cholesterol absorption existed between the NR and RT animals that could explain the contrasting cholesterolemic responses. When the daily amount of fecal cholesterol excreted was calculated we found that 90% of the fecal cholesterol was excreted within the first 5 days by the NR rabbits versus 93% by the RT animals (Fig. 4). Although the amount of cholesterol excreted by the RT group was somewhat greater during each of the 5 days, the daily difference was never significant ( $P < 0.05$ ). Similarly, there was no difference at any 24-h time interval over the total 9-day observation period between the NR and RT animals with regard to sitosterol excretion (Fig. 5). While all of the sitosterol was recovered by day-7, about 75% of the administered label was recovered by day-3 and more than 95% was recovered by day-6.

When the cumulative cholesterol absorbed over the first 6 days after the test meal was calculated, by using the sitosterol recovery data and expressing the results on a body weight basis, there was a small but significant difference ( $P < 0.05$ ) between the RT and NR groups (Table 1). Over the 6-day period the average amount of cholesterol absorbed from the test meal by the NR rabbits was  $22.00 \pm 3.13$  mg/kg body weight and  $18.30 \pm 2.35$  mg/kg body weight by the RT rabbits.

Fecal bile acid extractions were performed on aliquots of the daily fecal specimens used for the neutral sterol determinations. In contrast to the results obtained from the measurements of cholesterol excretion, there were significant differences between the amount of bile acids excreted by the NR and RT rabbits. On each of the 9 days that the samples were collected, there was a significantly larger amount of bile acids excreted by the RT animals Fig.

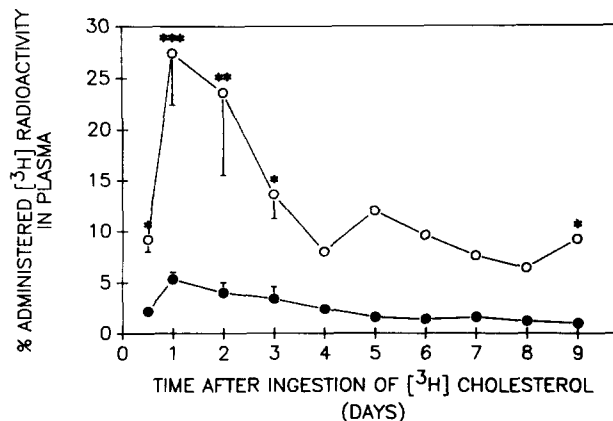
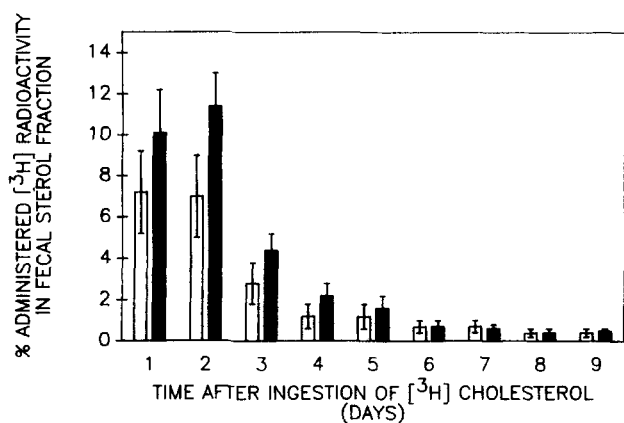
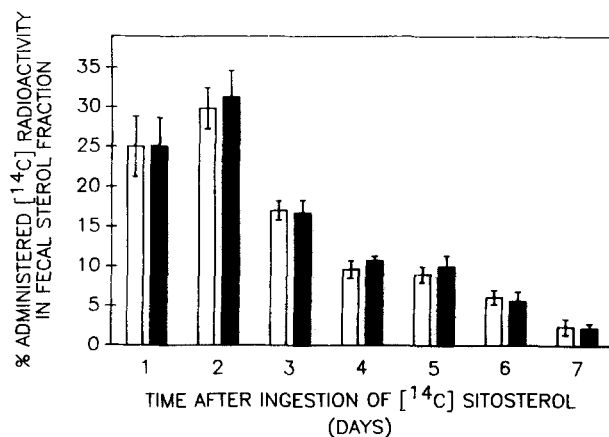


Fig. 3. Plasma  $^3\text{H}$  radioactivity after ingestion of a bolus of [ $^3\text{H}$ ]cholesterol by cholesterol-fed normoresponsive (hypercholesterolemic) (○) and resistant rabbits (●). When specimens from fewer than six animals per group were obtained only the group means are given, otherwise group means  $\pm$  SEM are shown; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; comparing normoresponsive rabbit values at designated time intervals with resistant rabbit values by unpaired  $t$ -test.



**Fig. 4.** Fecal excretion of  $^3\text{H}$  radioactivity in the neutral sterol fraction after ingestion of a bolus of [ $^3\text{H}$ ]cholesterol by normoresponsive and resistant rabbits. The results are expressed as means  $\pm$  SEM for the six animals per group. There was no significant difference ( $P > 0.05$ ) at any time interval, using the unpaired  $t$ -test, between the normoresponsive and the resistant groups.



**Fig. 5.** Fecal excretion of  $^{14}\text{C}$  radioactivity in the neutral sterol fraction after ingestion of a bolus of [ $^{14}\text{C}$ ]sitosterol by normoresponsive and resistant rabbits. The results are expressed as group means  $\pm$  SEM for the six animals per group. There was no significant difference ( $P > 0.05$ ) using unpaired  $t$ -test at any time interval between the normoresponsive and resistant groups.

6). The amounts of radioactive bile acids excreted peaked on day-2 in both groups of animals after ingestion of the test meal. Over the course of the entire 9 days there was more than a twofold greater ( $P < 0.0001$ ) amount of bile acids excreted by the RT animals than by the NR animals (Fig. 6).

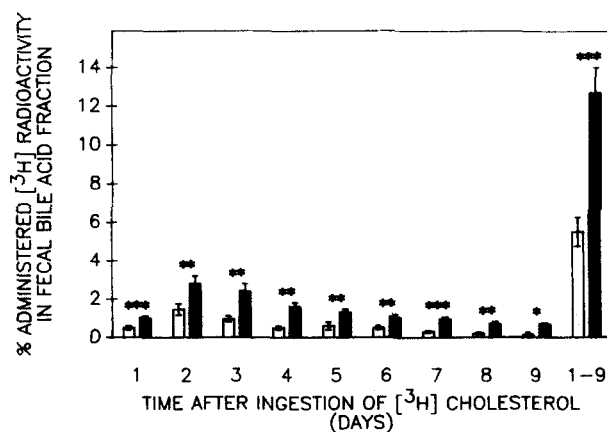
Separation and measurement by GC-MS of the fecal total neutral sterol and bile acid fractions were performed on samples of specimens collected on days 8 and 9 (Fig. 7). The major animal sterols were cholesterol and coprostanol, and the major bile acids were lithocholic and deoxycholic acid. Consistent with the data obtained from the bolus feeding studies, there was no significant difference between the mass of cholesterol or coprostanol excreted by the NR and RT groups on a daily basis. Although there was no difference between groups with respect to the mass of lithocholic acid excreted, there was a threefold increase in the amount of deoxycholic acid excreted by the RT as compared to the NR rabbits.

## DISCUSSION

In contrast to many species, typical NZW rabbits quickly develop exaggerated cholesterolemia and arteriosclerosis in response to cholesterol-enriched diets. The basis for the high susceptibility of these rabbits to dietary cholesterol appears to involve an ineffective homeostatic regulation of hepatic cholesterol metabolism. The liver of the typical rabbit, unlike that of some species, does not respond to an enlarged cholesterol pool by increasing cholesterol and bile acid fecal excretion. Instead, it responds with a decrease in cholesterol synthesis (30), an increase in cholesteryl ester-enriched VLDL secretion (31), and a decrease in the synthesis of LDL receptors

(32). As a result there is a decrease in the rate of VLDL remnant uptake, thus a rise of plasma VLDL cholesterol levels (31), and an enhanced conversion of VLDL to LDL, which results in a rise of plasma LDL (33).

The final pathways leading to the elimination of cholesterol from the body are largely by the direct secretion of cholesterol into the bile and the conversion, by liver parenchymal cells, of cholesterol to bile acids, which are subsequently excreted via the feces. The purpose of this study was to determine whether an enhanced fecal excretion of cholesterol or bile acids might occur in the hypercholesterolemia-resistant rabbit which in turn could help explain the resistance.



**Fig. 6.** Fecal excretion of  $^3\text{H}$  radioactivity in the bile acid fraction after ingestion of a bolus [ $^3\text{H}$ ]cholesterol by normoresponsive and resistant rabbits. The results are expressed as means  $\pm$  SEM for the six animals per group. The two bars to the right side of the figure represent the cumulative excretion over the 9-day observation period; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; comparing daily normoresponsive rabbit values with resistant rabbit values by unpaired  $t$ -test.

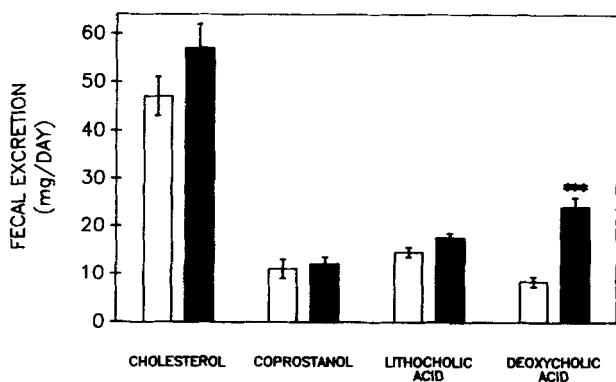


Fig. 7. Average daily fecal excretion of cholesterol, coprostanol, and the major bile acids (lithocholic and deoxycholic) by normoresponsive and resistant rabbits during days 8 and 9 of the cholesterol absorption studies. The results are expressed as means  $\pm$  SEM for the six animals per group; \*\*\* $P < 0.0001$ ; the absence of a symbol indicates no significant difference ( $P > 0.05$ ) using the *t*-test.

For accurate sterol balance data to be collected for input-output analysis of cholesterol homeostasis, a metabolic steady state is required. Although the test bolus was fed near the peak of the plasma cholesterol response curve, it is likely that the NR rabbits were continuing to accumulate cholesterol during this period, thus they would not have been in metabolic steady state and balance calculations would not have been valid.

Tritium was present in the plasma of both groups within 2 h after the oral consumption of the [ $^3\text{H}$ ]cholesterol test diet and reached peak activity at 24 h. At the first 24-h time interval there was more than a fivefold increase in the amount of label in the plasma of the NR animals versus the RT animals. The magnitude of this disparity persisted generally throughout the 9 days that the total plasma radioactivity was estimated. The methodologies used do not provide direct evidence about whether less dietary cholesterol reached the blood compartment of the RT rabbits, or whether the lower content was the result of a greatly increased clearance rate in the RT rabbits which had relatively much more contracted serum cholesterol pools. The absorption studies showing somewhat less cholesterol being absorbed by the RT animals suggests that less intestinal cholesterol reached the serum compartment. We have, however, also found that intravenously administered iodinated ( $^{125}\text{I}$ ) rabbit LDL and  $\beta$ -VLDL were each cleared from the serum of cholesterol-fed RT rabbits at a rate nearly three times greater than that at which NR animals cleared these lipoproteins (M. L. Overturf, S. Smith, and M. Soma, unpublished observations). Moreover, we have reported that incubated derepressed peripheral mononuclear cells from resistant rabbits provided with rabbit  $^{125}\text{I}$ -labeled LDL had about a 30% increase in the rate of uptake and degradation of LDL over that from normoresponsive rabbits (21).

Dietary cholesterol absorption was estimated, concurrently with the serum  $^3\text{H}$  measurements, to determine whether

a difference in absorption rate existed between the two groups of animals. The data indicated that there was a small but significant difference in the amount of cholesterol absorbed by the two groups when fed the test meal bolus. But the amount absorbed did not correlate with the plasma cholesterol levels. Cholesterol absorption was about 75% of that administered, which is in accordance with the results for typical rabbits reported by others (34). A much less efficient absorption of dietary cholesterol could have provided an explanation for the differences in plasma cholesterol concentrations between the NR and RT rabbits, but only a minimally significant difference ( $P < 0.05$ ) was found. Results similar to those now reported have been obtained from human studies comparing hypercholesterolemic (type II) patients with normal subjects (35, 36), and investigations of hypo- and hyper-responsive squirrel monkeys (37). There was no statistically significant difference ( $P < 0.05$ ) in cholesterol absorption between groups of either species.

A rapid disappearance of cholesterol from the serum of the resistant rabbits could indicate that it was being sequestered and accumulated by various tissues. However, as previously reported, NR rabbits fed the 0.1% cholesterol-enriched diet developed moderately severe aortic atherosclerosis with large accumulations of cholesterol and cholesteryl esters. By contrast, the RT animals never developed hypercholesterolemia and had no evidence of atherosclerotic vascular lesions (21).

In agreement with previous studies of typical rabbits (38, 39), the major neutral sterols found in the feces of both NR and RT rabbits were cholesterol and coprostanol. Although the ratios of quantities of cholesterol to coprostanol varied considerably between the animals of both groups, cholesterol was always the major neutral sterol excreted. Furthermore, since the bacterial conversion of cholesterol to coprostanol takes place almost exclusively in the terminal gastrointestinal tract, coprostanol is of minimal importance in the regulation of cholesterol metabolism.

Another potential means for maintaining relatively low plasma cholesterol levels during increased cholesterol consumption, as in the case of the resistant rabbits, would be increased cholesterol catabolism to bile acids concomitantly with increased excretion in the urine and feces. However, only small amounts of  $^3\text{H}$  label were found in the urine of the NR and RT animals, and the difference between the two groups was not significant. The low level of radioactivity excreted in the urine from ingested [ $^3\text{H}$ ]cholesterol was similar to the amount observed previously for typical rabbits (39); no urinary  $^{14}\text{C}$  activity was detected from either group. The RT rabbits, as compared to the NR rabbits, excreted significantly larger amounts of fecal bile acids. Furthermore, in preliminary tissue distribution studies, we have observed that 24 h after rabbits consumed 3 g of cholesterol-supplemented diet containing [ $4\text{-}^{14}\text{C}$ ]cholesterol their bile acid fractions (extracted from bile aspirated from the gallbladder at necropsy) contained an average of 2.2

times more radioactivity than specimens obtained from NR rabbits (M. L. Overturf and S. Smith, unpublished observations).

There is published evidence that one mechanism controlling plasma cholesterol in squirrel monkeys (37) and rabbits (34) is related to an increased rate of conversion of cholesterol to bile acids in the hyporesponsive animals. More recent studies of two inbred rabbit strains reported that hyperresponsive rabbits absorbed a greater percentage of dietary cholesterol than resistant rabbits did, and that fecal excretion of bile acids, but not neutral steroids, was increased in the hyporesponders (20). We found a somewhat greater cholesterol absorption rate by the normoresponsive (hyperresponsive) versus the resistant (hyporesponsive) animals. On the other hand, the difference in rates of bile acid excretion between our RT and NR animals was much greater than the relatively modest difference reported for JAX rabbits by Beynen et al. (20).

On the basis of the present and previous studies we propose that an overload of dietary cholesterol, delivered to the liver as chylomicron remnants in the RT rabbit, is quickly converted to bile acids. The conversion rate is thought to be so great that little  $\beta$ -VLDL is synthesized since none was found in the serum of cholesterol-fed RT rabbits (21). In the cholesterol-fed normoresponsive rabbits,  $\beta$ -VLDL likely suppressed apoB,E (LDL) receptor activity leading to an expansion of the serum cholesterol pool. By contrast, the serum cholesterol concentration remained remarkably unchanged in the resistant rabbits. Instead, LDL receptor activity of the RT rabbits would be expected to be maintained in a relatively up-regulated state and perhaps aid in the clearing of small quantities of cholesterol, as serum VLDL-cholesterol, that might have escaped conversion to bile acids (21). The potential mechanisms underlying the increased bile acid metabolism are currently being investigated.

In conclusion, accumulating evidence shows that while the cholesterolemic response to dietary cholesterol intake is highly variable between individuals of a species, some individuals and experimental animals are uniquely resistant. An increase in bile acid excretion has been identified as a potentially major means that may account for the heritable resistance to the development of dietary-induced hypercholesterolemia in our resistant rabbits. ■

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## REFERENCES

1. Shore, V. G., B. Shore, and R. G. Hart. 1974. Changes in apolipoproteins and properties of rabbit very low density lipoproteins on induction of cholesterolemia. *Biochemistry*. **13**: 1579-1587.
2. Roth, R. I., J. W. Gaubatz, A. M. Gotto, and J. R. Patsch. 1983. Effect of cholesterol feeding on the distribution of plasma lipoproteins and on the metabolism of apolipoprotein E in the rabbit. *J. Lipid Res.* **24**: 1-11.
3. Mahley, R. W., and K. S. Holcombe. 1977. Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. *J. Lipid Res.* **18**: 314-324.
4. Mahley, R. W., K. H. Weisgraber, and T. L. Innerarity. 1974. Canine lipoproteins and atherosclerosis. Characteristics of the plasma lipoproteins associated with atherogenic and non-atherogenic hyperlipidemia. *Circ. Res.* **35**: 722-733.
5. Lusk, L., J. Chung, and A. M. Scanu. 1982. Properties and metabolic fate of two very low density subfractions from rhesus monkey serum. *Biochim. Biophys. Acta* **710**: 134-142.
6. Nestel, P. N., T. Tada, T. Billington, M. Huff, and N. Fidge. 1982. Changes in very low density lipoproteins with cholesterol loading in man. *Metab. Clin. Exp.* **31**: 398-405.
7. Bersot, T. P., T. L. Innerarity, R. E. Pitas, S. C. Rall, K. H. Weisgraber, and R. W. Mahley. 1986. Fat feeding in human studies induces lipoproteins of density less than 1.006 that are enriched in apolipoprotein[a] and that cause lipid accumulation in macrophages. *J. Clin. Invest.* **77**: 622-630.
8. Pollak, O. J. 1945. Correlation between chemical and morphologic alterations in experimental atherosclerosis. *Arch. Pathol.* **39**: 11-18.
9. Clarkson, T. B., and H. B. Lofland. 1961. Effects of cholesterol-fat diets on pigeons susceptible and resistant to atherosclerosis. *Circ. Res.* **9**: 106-109.
10. Morrisett, J. D., H-S. Kim, J. R. Patsch, S. K. Datta, and J. J. Trentin. 1982. Genetic susceptibility and resistance to diet-induced atherosclerosis and hyperlipoproteinemia. *Arteriosclerosis*. **2**: 312-324.
11. Clarkson, T. B., J. R. Kaplan, and M. R. Adams. 1985. The role of individual differences in lipoprotein, artery wall, gender and behavioral responses in the development of atherosclerosis. *Ann. NY Acad. Sci.* **454**: 28-45.
12. Katan, M. B., and A. C. Beynen. 1987. Characteristics of human hypo- and hyperresponders to dietary cholesterol. *Am. J. Epidemiol.* **125**: 387-399.
13. Clarkson, T. B., H. B. Lofland, B. C. Bullock, and H. O. Goodman. 1971. Genetic control of plasma cholesterol. Studies on squirrel monkeys. *Arch. Pathol.* **92**: 37-45.
14. Imai, Y., and H. Matsumura. 1973. Genetic studies on induced and spontaneous hypercholesterolemia in rats. *Atherosclerosis*. **18**: 59-64.
15. Roberts, D. C. K., C. E. West, T. G. Redgrave, and J. B. Smith. 1974. Plasma cholesterol concentration in normal and cholesterol-fed rabbits. Its variation and heritability. *Atherosclerosis*. **19**: 369-380.
16. Takeuchi, N., M. Ito, and Y. Yamamura. 1976. Cholesterol metabolism in rats sensitive to high cholesterol diet. *Adv. Exp. Med. Biol.* **67**: 267-288.
17. McGill, H. C., C. A. McMahan, G. E. Mott, Y. N. Marinéz, and T. J. Keuhl. 1988. Effects of selective breeding on the cholesterolemic responses to dietary saturated fat and cholesterol in baboons. *Arteriosclerosis*. **8**: 33-39.
18. Adams, W. C., E. M. Gaman, and A. S. Feigenbaum. 1972. Breed differences in the responses of rabbits to atherogenic diets. *Atherosclerosis*. **16**: 405-411.
19. Van Zutphen, L. F. M., and R. R. Fox. 1977. Strain differences in response to dietary cholesterol by JAX rabbits: correlation with esterase patterns. *Atherosclerosis*. **28**: 435-446.
20. Beynen, A. C., G. W. Meijer, A. G. Lemmens, J. F. C. Glatz, A. Versluis, M. B. Katan, and L. F. M. Van Zutphen. 1989. Sterol balance and cholesterol absorption in inbred strains of rabbits hypo- or hyperresponsive to dietary cholesterol. *Atherosclerosis*. **77**: 151-157.



21. Overturf, M. L., S. A. Smith, D. Hewett-Emmett, D. S. Loose-Mitchell, M. R. Soma, A. M. Gotto, Jr., and J. D. Morrisett. 1989. Development and partial metabolic characterization of a dietary cholesterol-resistant colony of rabbits. *J. Lipid Res.* **30**: 263-273.
22. Borgström, B. 1969. Quantification of cholesterol absorption in man by fecal analysis after the feeding of a single isotope-labeled meal. *J. Lipid Res.* **10**: 331-337.
23. Grundy, S. M., E. H. Ahrens, Jr., and T. A. Miettinen. 1965. Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* **6**: 397-410.
24. Grundy, S. M., E. H. Ahrens, and G. Salen. 1968. Dietary  $\beta$ -sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies. *J. Lipid Res.* **9**: 374-387.
25. Miettinen, T. A., E. H. Ahrens, Jr., and S. M. Grundy. 1968. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral sterols. *J. Lipid Res.* **6**: 411-424.
26. Marai, L., and A. Kuksis. 1982. Gas chromatographic-mass spectrometric identification of n-butylated products of some common keto carboxylic acids. *J. Chromatogr.* **249**: 359-368.
27. Child, P., M. Aloe, and D. Mee. 1987. Separation and quantitation of fatty acids, sterols, and bile acids in feces by gas chromatography as the butyl ester-acetate derivatives. *J. Chromatogr.* **415**: 13-26.
28. Kuksis, A., J. J. Myher, L. Marai, and K. Geher. 1975. Determination of plasma lipid profiles by automated gas chromatography and computerized data analysis. *J. Chromatogr. Sci.* **13**: 423-430.
29. Armin, J., R. T. Grant, H. Pels, and E. B. Reeve. 1952. The plasma, cell and blood volumes of albino rabbits as estimated by the dye (T 1824) and  $^{32}\text{P}$  marked cell methods. *J. Physiol. (London)* **116**: 59-73.
30. Kovanen, P. T., J. L. Goldstein, and M. S. Brown. 1978. High levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and cholesterol synthesis in the ovary of the pregnant rabbit. *J. Biol. Chem.* **253**: 5126-5132.
31. Daugherty, A., G. Schonfeld, B. E. Sobel, and L. G. Lange. 1986. Metabolism of very low density lipoproteins after cessation of cholesterol feeding in rabbits. A factor potentially contributing to the slow response of atheromatous plaques. *J. Clin. Invest.* **77**: 1108-1115.
32. Kovanen, P. T., M. S. Brown, S. K. Basu, D. W. Bilheimer, and J. L. Goldstein. 1981. Saturation and suppression of hepatic lipoprotein receptors: a mechanism for the hypercholesterolemia of cholesterol-fed rabbits. *Proc. Natl. Acad. Sci. USA.* **78**: 1396-1400.
33. Goldstein, J. L., and M. S. Brown. 1984. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. *J. Lipid Res.* **25**: 1450-1462.
34. Massaro, E. R., and D. B. Zilversmit. 1977. Controlling factors in the maintenance of plasma cholesterol concentration in the rabbit. *J. Nutr.* **107**: 596-605.
35. Connor, W. E., and D. S. Lin. 1974. The intestinal absorption of dietary cholesterol by hypercholesterolemic (Type II) and normocholesterolemic humans. *J. Lab. Clin. Invest.* **53**: 1062-1070.
36. Lin, D. S., and W. E. Connor. 1980. The long term effects of dietary cholesterol upon the plasma lipids, lipoproteins, cholesterol absorption, and sterol balance in man: the demonstration of feedback inhibition of cholesterol biosynthesis and increased bile acid excretion. *J. Lipid Res.* **21**: 1042-1052.
37. Lofland, H. B., Jr., T. B. Clarkson, R. W. St. Clair, and N. D. M. Lehner. 1972. Studies on the regulation of plasma cholesterol levels in squirrel monkeys of two genotypes. *J. Lipid Res.* **13**: 39-47.
38. Hellström, K. 1965. On the bile acid and neutral fecal steroid excretion in man and rabbits following cholesterol feeding. *Acta Physiol. Scand.* **63**: 21-35.
39. Huff, M. W., and K. K. Carroll. 1980. Effects of dietary protein on turnover, oxidation, and absorption of cholesterol, and on steroid excretion in rabbits. *J. Lipid Res.* **21**: 546-558.