Identification of a metabolic difference accounting for the hyper- and hyporesponder phenotypes of cynomolgus monkey

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Abstract These studies were done to determine whether an underlying metabolic difference could account for the higher concentration of cholesterol carried in low density lipoproteins (LDL-C) in male hyperresponder (HR) cynomolgus monkeys than in their hyporesponder (HO) counterparts during dietary cholesterol challenge. All animals were fed to steady state at 5 months a diet that had a constant concentration of cholesterol (0.19 mg/g), triacylglycerol (175 mg/g), and soluble fiber. There were no differences in these two phenotypes with respect to the profile of fatty acids in the liver and bile acids in the gallbladder, or in the relationship of cholesterol synthesis to cholesteryl ester formation in the liver. The rate of cholesterol synthesis in all extrahepatic tissues was also the same in the HO and HR animals but was 2.1 mg/day per kg body weight less in the liver of the HR monkeys. When challenged with a greater dietary cholesterol load, therefore, the HR animal could not readily further down-regulate synthesis and so shifted more cholesterol into the ester pool (9.4 mg/g) than did the HO animal (3.9 mg/g). Also the LDL-C concentration was more markedly elevated (412 mg/dl) compared to the hyporesponder monkey (188 mg/dl). Thus, this single metabolic alteration apparently accounted for the HO and HR phenotypes. As this difference was not due to variation in the delivery of sterol from the extrahepatic organs to the liver, it must reflect a difference in either net intestinal sterol absorption or net hepatic sterol excretion in the two phenotypes.—**Turley, S. D., D. K. Spady, and J. M. Dietschy.** Identification of a metabolic difference accounting for the hyper- and hyporesponder phenotypes of cynomolgus monkey. *J. Lipid Res.* 1997. **38:** 1598–1611.

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The concentration of cholesterol carried in plasma low density lipoproteins (LDL-C) in the steady state is determined by the interplay of environmental factors such as the content of sterol, triacylglycerol, and soluble fiber in the diet, with genetically determined polymorphisms in one or more of the several dozen transporters, enzymes, and apoproteins involved in maintenance of cholesterol homeostasis in the whole animal or human. In most animals, including humans, the majority of cholesterol synthesis takes place in the extrahepatic organs that make up >95% of body mass (1–3). While the rate of hepatic cholesterol synthesis is usually low, it can increase to compensate for excessive sterol loss in the feces as is seen, for example, when the content of dietary fiber is increased (4, 5). Alternatively, when cholesterol inflow from the diet is increased, hepatic synthesis is suppressed. Thus, the liver and, to a much lesser degree, intestine uniquely respond to changes in net cholesterol balance across the whole animal (6, 7). In contrast, synthesis in the other extrahepatic organs remains essentially unaffected by these changes in net sterol balance.

In these same species, the liver also plays the central role in the production of very low density lipoproteins (VLDL) and, ultimately, LDL and in the removal of LDL-C from the plasma (2). In the normal animal and human, LDL-C uptake and degradation takes place by both a receptor-dependent process and a receptor-independent process (2, 8). While much of receptor-independent LDL-C transport is located in the extrahepatic tissues, approximately 90% of receptor-dependent transport is found in the liver (3, 9–12). The activity

Abbreviations: LDL-C, cholesterol in low density lipoprotein; VLDL, very low density lipoprotein; LDLR, LDL receptor; J_0, LDL-C production rate; J^m, maximal rate of receptor-dependent LDL-C transport; $*K_m$, concentration of LDL-C necessary to give half the value of J^m; P, rate constant for receptor-independent LDL-C transport; apoB, apolipoprotein B; HO, hyporesponder phenotype; HR, hyperresponder phenotype; bw, body weight; TC, total plasma cholesterol; DPS, digitonin-precipitable sterols; ACAT, acyl CoA:cholesterol acyltransferase; HDL, high density lipoprotein.

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of these hepatic LDL receptors (LDLR) appears to be regulated independently of the regulation of hepatic cholesterol synthesis (7, 13, 14). Thus, when net cholesterol delivery to the liver is increased, there is suppression of hepatic cholesterol synthesis. If this suppression is adequate to balance the increased sterol acquisition from the intestine, there is no expansion of the pool of excess cholesterol in the hepatocyte and no down-regulation of LDLR activity. If, on the other hand, suppression of synthesis to virtually zero is inadequate to offset the inflow of dietary sterol, then a new steady state is eventually reached where the pool of excess sterol in the liver cell is expanded and there is suppression of LDLR activity (15, 16). The distribution of this excess cholesterol between the regulatory and ester pools is also profoundly influenced by the types of individual fatty acids that enrich the lipid pools of the liver. Under conditions where cholesterol balance across the liver is constant, fatty acids like the 18:1(9*c*) compound sequester sterol into the ester pool and increase LDLR activity while lipids such as the 16:0 fatty acid have the opposite effect (17, 18).

Thus, in any animal or human, the concentration of LDL-C that is achieved at steady state is dictated by four variables, each of which can be independently measured (8, 11). These are *1*) the rate of LDL-C production (J_t) which reflects the rate of VLDL-C secretion by the liver and its ultimate conversion to LDL-C; *2*) the maximal rate of receptor-dependent LDL-C transport (J^m) which essentially mirrors LDLR number in the liver; *3*) the apparent Michaelis constant for the LDLR ($*K_m$) which is determined by both the absolute affinity of LDL for its receptor and the competition exerted by other ligands with the LDL particle for this same receptor; and *4*) the rate of receptor-independent uptake (P) (8, 19). Clearly both environmental factors and genetic variability in the proteins involved in sterol homeostasis can affect one or more of these rate constants and, therefore, steady-state plasma LDL-C levels.

Two major environmental factors have been identified that are important in this regard. The first is the magnitude of net cholesterol balance across the liver. Increasing the dietary cholesterol load in virtually any animal is associated with progressive suppression of J^m, an increase in J_t, but no alteration in either $*K_m$ or P (20, 21). The second is the profile of fatty acids within the hepatic lipid pools. Feeding triacylglycerol rich in unsaturated fatty acids increases J^m and reduces J_t while certain saturated lipids have the opposite effect (16, 17, 21). The changes in the plasma LDL-C levels brought about by these fatty acids occur under conditions where there is neither a demonstrable alteration in sterol balance across the liver nor a change in $*K_m$ or P. Several genetic polymorphisms have also been identified that influence these rate constants. For example, amino acid substitutions in the apoprotein (apo) B and E molecules have been described that alter the affinity of the lipoproteins containing these ligands for the LDLR. Thus, in humans with either familial defective apoB disease (22, 23) or the apoE-4 phenotype (24–26) the value of $*K_m$ is elevated so that the steady-state concentration of LDL-C is higher at any level of J^m, J_t, and P than would be the case if these mutations were not present (19).

Unfortunately, while a few of these genetic polymorphisms have been described, in other instances there is little understanding of the metabolic differences that account for variability in these rate constants, and, therefore, the plasma LDL-C levels, in individual animals or humans. Intake of an increased load of dietary cholesterol, for example, nearly always leads to elevation of the LDL-C concentration; however, in virtually all outbred species it is possible to identify individuals where the increase in the plasma cholesterol level is either much less (hyporesponder, HO) or much greater (hyperresponder, HR) than the average response. This is true in various types of monkeys, rabbits, hamsters, rats, and other species (27–34). Because of the major importance of this type of polymorphism, the present studies were undertaken in the outbred cynomolgus monkey in which LDL metabolism has already been described in detail and where many aspects of cholesterol metabolism are quantitatively similar to those in humans (3, 35).

Thus, for example, on a dietary cholesterol intake of 5.4 mg/day per kg body weight (bw), this animal synthesizes about 11 mg of cholesterol per day per kg bw, and 89% of this synthesis takes place in the extrahepatic organs. Approximately 17 mg/day per kg bw of LDL-C is formed, and 80% of this lipoprotein is cleared from the plasma by the liver. Importantly, in this species, as in the human, dietary cholesterol challenge specifically raises the concentration of LDL-C while there is little change in the concentration of the other major lipoproteins (3). Using the male cynomolgus monkey, therefore, three groups of studies were undertaken where *1*) the HO and HR phenotypes were much more precisely defined, *2*) net sterol balance across the major tissue compartments of the HO and HR animals was measured under conditions where dietary cholesterol intake approximated that of humans, and *3*) the differences in the intrahepatic metabolism of cholesterol were defined in the two phenotypes under the stress of an increased dietary sterol challenge. These studies revealed that virtually all aspects of cholesterol metabolism were quantitatively similar in the HO and HR

animals except that there was a demonstrable difference in net cholesterol balance across the liver in the two groups that could fully explain the two phenotypes.

MATERIALS AND METHODS

Animals and diets

Feral adult, male cynomolgus monkeys (*Macaca fasicularis*) that had been screened for their responsiveness to dietary cholesterol were obtained from the primate centers at the Bowman Gray School of Medicine (Winston-Salem, NC) and the Winters Institute (Winters, CA), and housed as previously described (3). On commercial, low-fat primate diet, these animals had plasma total cholesterol (TC) concentrations of 80–120 mg/dl. As previously described, the experimental diets were custom formulated and of constant composition with respect to total lipids (17.5 g per 100 g of diet) and soluble fiber, but of variable composition with respect to cholesterol content (3). The critical measurements of sterol balance were all made in animals maintained on a diet containing 0.19 mg/g (0.044 mg/kcal) of cholesterol and designated in the table and figures as the low cholesterol diet. This diet was designed to provide the animals with a daily sterol intake essentially equal to that of a human on a relatively low cholesterol intake (~5 mg/day per kg bw). In other experiments, individual animals were also fed diets containing increased amounts of sterol up to a maximum of 1.9 mg/g (0.44 mg/kcal). This latter diet, which provided the maximal cholesterol challenge, was designated as the high cholesterol diet.

Before beginning any experiments, it was necessary to establish the phenotype of the individual animals with respect to the response of their plasma TC concentration when challenged with a large amount of dietary cholesterol. To define the phenotype, each animal was fed the high cholesterol diet for 9 weeks before the plasma cholesterol was measured. Animals with a TC concentration of >350 mg/dl were tentatively designated as hyperresponders (HR) while those with a concentration of <250 mg/dl were defined as hyporesponders (HO). After this dietary challenge, all animals were returned to the low cholesterol diet until entered into specific experimental protocols. While the cholesterol challenge elevated both the plasma LDL-C concentration and the level of hepatic cholesteryl esters, as shown in **Fig. 1,** after 2 months on the low cholesterol diet both of these values had returned essentially to control levels, and there was no further decrease in either

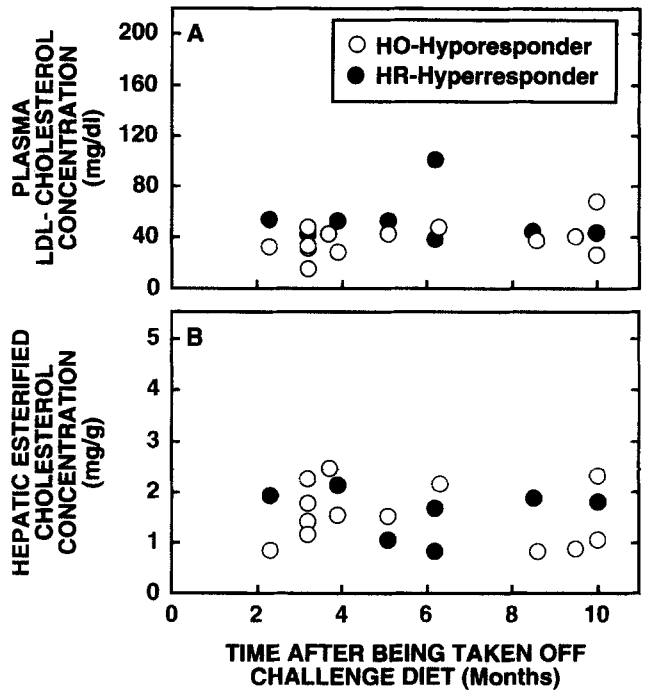

Fig. 1. Plasma LDL-C and hepatic cholesteryl ester concentrations in hyporesponder (HO) and hyperresponder (HR) animals after dietary cholesterol challenge. In order to define the phenotype of these monkeys, each animal had been placed on the high cholesterol diet for 9 weeks and identified as an HO or HR animal. All monkeys were then returned to the low cholesterol diet and followed for 2 to 10 months during which time the plasma LDL-C and hepatic cholesteryl ester concentrations were measured in individual animals. Each data point represents the results found in a single monkey during this re-equilibration to the low cholesterol diet.

value in the HO or HR animals, even when followed on this diet for up to 10 months. Thus, all measurements were made after the animals had been on the appropriate experimental diets for 5 months by which time the monkeys had turned over approximately 150 pools of LDL-C and had reached new steady states in terms of hepatic cholesterol levels and synthesis rates, and plasma total and LDL-C concentrations (3). In a single comparative study, male hamsters were also fed variable amounts of cholesterol to steady state at 30 days. All experiments were approved by the Institutional Animal Care and Research Advisory Committee.

Measurement of rates of sterol synthesis in vivo and in vitro

The rates of hepatic, intestinal, carcass, and whole body sterol synthesis were measured in vivo using [^{3}H]water as detailed elsewhere (1, 3, 36). The rates of sterol synthesis were expressed in terms of tissue [^{3}H]DPS content, and represent the nmol or μmol of [^{3}H]water incorporated into digitonin-precipitable sterols (DPS) per h per g of tissue (nmol/h per g) or per

whole organ (μmol/h per whole organ). The rates of whole body synthesis were calculated as the sum of the whole organ [^{3}H]DPS contents normalized per kg body weight (μmol/h per kg bw). When the liver and small intestine were removed from the monkeys that had been administered [^{3}H]water, pieces of both organs were immediately sliced and incubated in Krebs bicarbonate buffer containing [1-^{14}C]octanoate (New England Nuclear, Boston, MA) at a concentration of 1.5 mM. The flux of C_2 units into DPS was then determined in vitro (36, 37) These data were expressed as the nmol of acetyl CoA units, i.e., C_2 units, incorporated into DPS per h per g of tissue (nmol/h per g).

Analytical procedures

The methods for measuring plasma total and lipoprotein cholesterol concentrations, fatty acid composition of diets and tissues, and biliary bile acid composition are described in detail elsewhere (3). The esterified and unesterified cholesterol fractions in the liver were separated on silicic acid/celite columns and their cholesterol content was then measured by GLC (3, 38, 39).

Analysis of data

The equations used for calculating the specific activities of plasma water, the rates of incorporation of either [^{3}H]water or C_2 units from [1-^{14}C]octanoate into DPS, and for converting the [^{3}H]DPS content of the whole animal to an equivalent mass of newly synthesized cholesterol, are described elsewhere (1, 3, 36). Data are presented as the mean ± 1 SEM for the number of animals specified for each group. Differences between mean values were tested for statistical significance using an unpaired, two-tailed, Student's *t*-test that took into account both the equality of variance and the normality of distribution of the data. Two sets of comparisons were made. One compared the values for HO and HR animals within either the low or the high dietary cholesterol groups. For this comparison statistically significant differences ($P < 0.05$) are denoted by (*). The other set of comparisons was between values for a particular phenotype, i.e., the HO or HR animals, on the low and high cholesterol diets. In this case, statistically significant differences ($P < 0.05$) are denoted by (‡).

RESULTS

Definition of the HO and HR phenotypes

During the initial 9 weeks screening of nearly 200 animals on the high cholesterol diet, approximately two-thirds of the monkeys achieved intermediate TC concentrations in the range of 250 to 350 mg/dl. In the remaining one-third of the animals, the plasma cholesterol response to this dietary cholesterol challenge was either much lower (<250 mg/dl) or substantially higher (>350 mg/dl), and these two groups of animals, therefore, were tentatively identified as hyporesponders (HO) and hyperresponders (HR), respectively. Subsequently, each of these animals was placed on a specific experimental diet for 5 months. It is well established that dietary sterol, fatty acids, and soluble fiber all profoundly affect the distribution of cholesterol between the regulatory and ester pools in the liver and so alter hepatic LDL receptor activity and the LDL-C production rate (2, 17, 18, 20, 40). To simplify these studies, however, the fatty acid composition, total triacylglycerol content, and fiber content of the experimental diets were kept constant. In addition, the critical measurements of sterol balance were all made under conditions where the diet also contained a constant concentration of dietary cholesterol that approximated that in the human diet. All animals were hand fed 16 g per kg bw of the experimental diets twice each day for 5 months and, on average, ~11% was wasted in all experimental groups. Thus, the animals receiving the low cholesterol experimental diet had a dietary sterol intake of 5.4 mg/day per kg bw, which is virtually identical to the sterol intake of Western humans on moderately low cholesterol diets. In some experiments other animals were challenged with diets containing higher concentrations of cholesterol up to a maximum of 54 mg/day per kg bw.

Characterization of fatty acid content and the handling of excess dietary cholesterol within the liver of the two phenotypes

Regulation of both the rate of cholesterol synthesis and the level of LDL receptor activity in the liver is influenced by the inflow of dietary fatty acids and sterol (2, 17, 20). Clearly, the differences observed in the response of the plasma cholesterol concentration to dietary cholesterol challenge in the two phenotypes might be explained in terms of genetic polymorphisms in one or more of the enzymes or transporters that determine the types of fatty acid in the liver or the distribution of cholesterol between the regulatory and ester pools of the hepatocyte.

As the profile of fatty acids in the liver cell is one of the most powerful determinants of the distribution of cholesterol between the regulatory and ester pools (17, 18), the first study undertaken was to determine whether the composition of this pool varied in the HO and HR monkeys after feeding diets of identical fatty acid composition. As shown in **Fig. 2A,** the diets fed for 5 months had a constant composition of lipids where

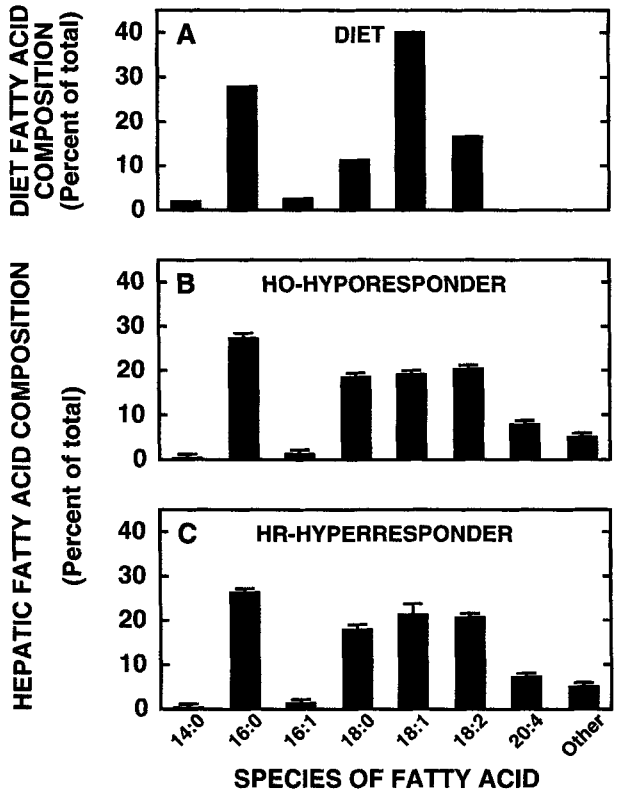

Fig. 2. Hepatic fatty acid composition in hyporesponder (HO) and hyperresponder (HR) monkeys fed different levels of dietary cholesterol for 5 months. At the end of this feeding period the steady-state concentration of the various fatty acids in the total lipid fraction of the liver was determined. This figure shows the distribution of fatty acids in the diet (A) that was consumed for 5 months and in the livers of the HO and HR animals (B, C). As the composition of fatty acids did not change with different levels of dietary cholesterol intake, these values have been combined in each of the experimental groups. Each bar represents the mean ± 1 SEM for data from 8 HO or 7 HR monkeys. There were no significant differences between the HO and HR animals.

the 18:1(9*c*), 16:0, 18:2(9*c*,12*c*) and 18:0 fatty acids were present in the highest concentrations. In this study, individual animals received diets containing variable amounts of cholesterol (from 5.4 to 54 mg/day per kg bw) over the 5-month experimental period. However, as the fatty acid profile in the liver did not vary with the level of dietary cholesterol challenge, the results obtained in individual animals in the HO and HR groups were combined and are shown in panels B and C, respectively. As is apparent, the profile of fatty acids in the livers of the two phenotypes was virtually identical. Therefore, the difference in the response of the plasma cholesterol concentration to dietary sterol challenge in these two groups could not be explained, for example, by a genetically determined enrichment or depletion of a fatty acid in the liver known to alter acyl CoA:cholesterol acyltransferase (ACAT) activity and, therefore, LDL-C metabolism (17, 18).

Secondly, it is also known, as shown in **Fig. 3A,** that in the steady state excess cholesterol entering the liver is not shifted into the regulatory pool affecting LDL receptor activity or into the ester pool until hepatic sterol synthesis is suppressed nearly to zero. In this example, carried out in the hamster, individual animals were fed increasing amounts of cholesterol until new steady states were achieved (at 30 days in this species). As is apparent, there was no expansion of the sterol pools in the liver until the amount of cholesterol fed to each animal exceeded the capacity of the liver to compensate

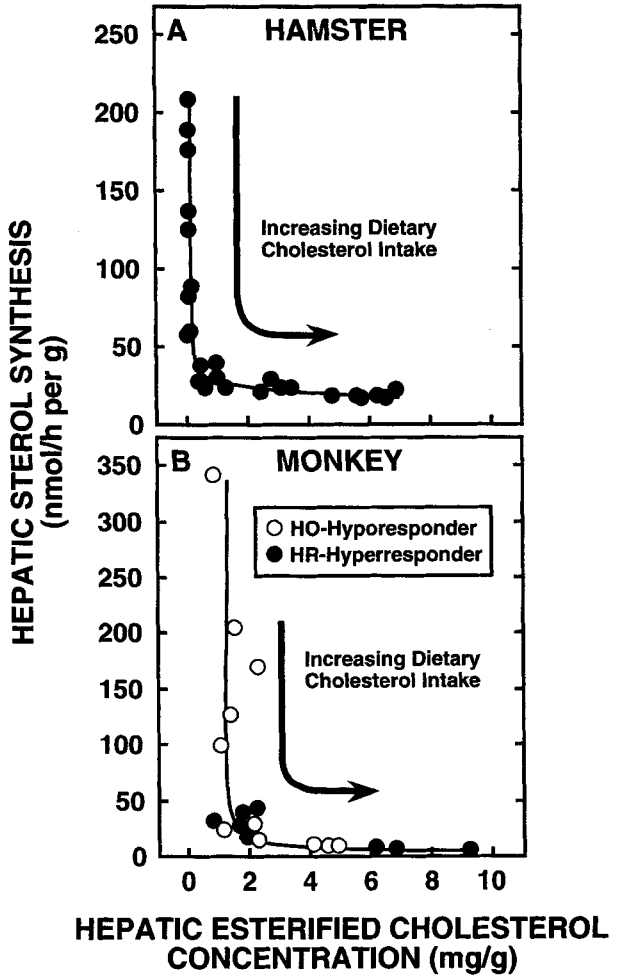

Fig. 3. Relationship between hepatic cholesterol synthesis and the appearance of excess cellular sterol in HO and HR monkeys fed different amounts of dietary cholesterol for 5 months. For purposes of comparison, panel A shows this relationship in individual hamsters fed increasing amounts of cholesterol to steady state at 30 days. Panel B shows similar experiments in HO and HR monkeys fed variable amounts of cholesterol for 5 months. In both cases, the arrows indicate animals receiving increasing dietary cholesterol loads. Each point represents a single animal. Data are shown for 25 hamsters (A) and 11 HO and 8 HR monkeys (B).

by decreasing synthesis. This characteristic behavior has been found in all species that have been tested and, as shown in panel B, was also found in these monkeys. In these 19 animals, each of which was fed a different amount of cholesterol for 5 months, no expansion of the intracellular sterol ester pool was evident in either the HO or HR animals until the rate of synthesis approached zero. It was apparent in this preliminary study, however, that the HR animals had lower rates of hepatic sterol synthesis on the lower cholesterol diets and achieved much higher levels of cholesteryl esters when challenged with the diets containing greater amounts of sterol. While this difference will be explored in detail later, these studies demonstrated that the HO and HR animals manifested the same relationship between steady-state rates of cholesterol synthesis and cholesteryl ester concentration in the liver as was manifest in normal animals. Therefore, the difference in these two phenotypes could also not be explained, for example, by inappropriate shifts of cholesterol into the ester pool in the HO monkeys.

A third possibility existed, nevertheless, that once sterol synthesis was suppressed nearly to zero there was a different distribution of cholesterol between the unesterified and esterified pools in the livers of the HO and HR animals when challenged with further increases in the amount of dietary cholesterol. As shown in **Fig. 4,** however, once hepatic synthesis reached zero and expansion of the intrahepatic sterol pools did take place, the distribution of cholesterol between the esterified and unesterified pools was essentially identical in 26 HO and 18 HR monkeys. There was no significant difference between the linear regression curves fitted to the data obtained in the two phenotypes. These results further suggested that the response of the plasma LDL-C concentration in the HO and HR animals could not be attributed, for example, to a subtle polymorphism affecting the apparent Michaelis constants for the cholesterol esterification/hydrolase system in the liver cell.

Finally, the data in **Fig. 5** indicate that there were also no apparent differences in the HO and HR animals with respect to their ability to synthesize and reabsorb specific bile acids. The absolute concentration of bile acids (242–252 μmol/ml), phospholipid (26–28 μmol/ml), and cholesterol (4–8 μmol/ml) in gallbladder bile was virtually identical in the two phenotypes. This was also true when these data were expressed as molar percentages. More importantly, however, as shown in this figure, there was also no significant difference in the relative concentration of any of the major bile acids in the gallbladder bile of the two groups of monkeys. These data indicated that the difference in the responsiveness of the two groups of animals could also not be attributed to a difference in the relative hydrophobicity of

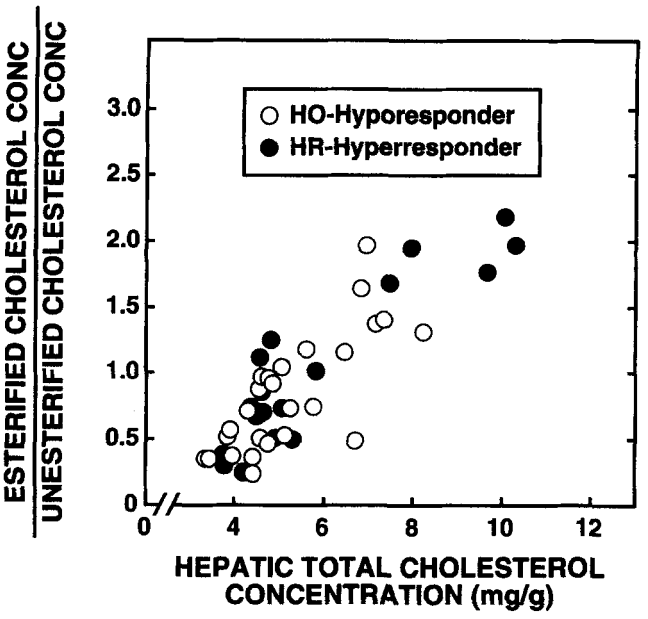

Fig. 4. Relationship of esterified to unesterified cholesterol in the liver of HO and HR monkeys fed different amounts of cholesterol for 5 months. At the end of this feeding period, the concentration of esterified and unesterified cholesterol was measured in the liver of each animal. Each point represents the ratio of these values found in an individual animal. Data for a total of 26 HO and 18 HR monkeys are presented. The slope of the linear regression lines calculated for each set of points by the method of least squares was essentially the same for the HO ($y = -0.52 + 0.26x$, $r = 0.76$) and HR ($y = -0.50 + 0.26x$, $r = 0.91$) animals.

the bile acid pool secreted into the small intestine that might differentially affect either LDL receptor activity or the percentage of dietary cholesterol absorbed (41, 42).

Thus, based upon these four experiments, the phenotype HR was assigned to an animal that elevated its plasma TC to >350 mg/dl (LDL-C > 280 mg/dl) when fed the high cholesterol challenge diet for 9 weeks, while the HO designation was reserved for the animal that raised its plasma TC to a value of <250 mg/dl (LDL-C < 200 mg/dl). Furthermore, the HR and HO animals were shown to have no differences in hepatic fatty acid (Fig. 2) and bile acid (Fig. 5) profiles, and to exhibit no apparent polymorphisms in the proteins and transcription factors that dictated the relationship between synthesis and excess cellular cholesterol (Fig. 3) or between esterified and unesterified cholesterol (Fig. 4) once the steady state was reached.

Characterization of net sterol balance across the liver of the HO and HR phenotypes

As these four experiments revealed no apparent differences in the intrahepatic handling of fatty acid or cholesterol in the HO and HR animals, the next major

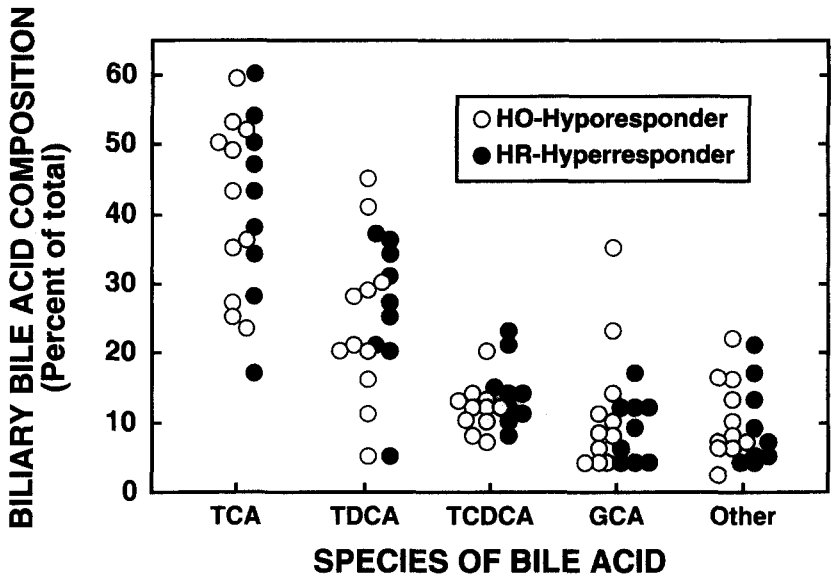

Fig. 5. Relative concentrations of the major bile acids in gallbladder bile of HO and HR monkeys fed different amounts of cholesterol for 5 months. Gallbladder bile was aspirated from 11 HO and 9 HR monkeys 5 months after they had been fed variable amounts of dietary cholesterol. These bile samples were then analyzed for their relative concentrations of the major bile acids, taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), and glycocholic acid (GCA). Five other bile acids (glycodeoxycholic acid, glycochenodeoxycholic acid, glycoursodeoxycholic acid, taurolithocholic acid, and tauroursodeoxycholic acid) were present in minor amounts in all animals, and these were combined in a single group. Each point represents the percentage value found in an individual animal. There were no significant differences between the mean values in the HO and HR animals.

study explored the possibility that the HO and HR phenotypes were explicable in terms of a difference in hepatic sterol balance in the two groups of monkeys. These critical measurements were made in HO and HR animals fed the low cholesterol diet to steady state at 5 months. As illustrated in **Table 1,** during the initial characterization of the 10 animals entered into this study, the HR and HO animals reached very different TC concentrations (478 and 162 mg/dl, respectively) when challenged with the high cholesterol diet (column A). However, after being fed the low cholesterol diet to steady state at 5 months, there was no significant difference in the plasma TC (B), VLDL-C (C), LDL-C (D), or HDL-C (E) concentrations in the two groups. Of particular importance, the concentration of hepatic cholesteryl esters in the liver of the HO (1.4 ± 0.3 mg/g) and HR (1.7 ± 0.2 mg/g) monkeys also was indistinguishable.

Under these carefully established conditions where the dietary cholesterol intake equalled 5.4 mg/day per

TABLE 1. Plasma lipoprotein cholesterol concentrations in hypo- and hyperresponder monkeys fed a low or high cholesterol diet

				Plasma Total Cholesterol Concentration		Plasma Lipoprotein Cholesterol Concentrations at Time of Study		
Phenotype	Diet	Number of Animals	Final Body Weight	A. At End of Challenge Diet	B. At Time of Study	C. VLDL-C	D. LDL-C	E. HDL-C
			kg	*mg/dl*			*mg/dl*	
HO	Low cholesterol	5	5.6 ± 0.3	162 ± 10	103 ± 12	3 ± 1	40 ± 8	60 ± 5
HR	Low cholesterol	5	5.7 ± 0.2	478 ± 37*	127 ± 19	6 ± 1	57 ± 11	64 ± 9
HO	High cholesterol	4	5.9 ± 0.6	158 ± 17	243 ± 51‡	10 ± 2‡	188 ± 49‡	45 ± 3‡
HR	High cholesterol	4	5.1 ± 0.2	499 ± 76*‡	499 ± 73*‡	46 ± 16‡	412 ± 62*‡	41 ± 7

Groups of hypo- (HO) and hyperresponder (HR) monkeys were fed either a low or a high cholesterol diet for 5 months at which time they were used for sterol synthesis experiments as described in Materials and Methods. Plasma collected on the day of study was separated by density gradient ultracentrifugation into three fractions corresponding to VLDL (d < 1.020 g/ml), LDL (d 1.020-1.063 g/ml) and HDL (d > 1.063 g/ml). Each value represents the mean ± 1 SEM of data obtained in the number of animals indicated.

*$P < 0.05$ for comparison with hyporesponder (HO) animals fed the same diet.

‡$P < 0.05$ for comparison with animals of the same phenotype on the low cholesterol diet.

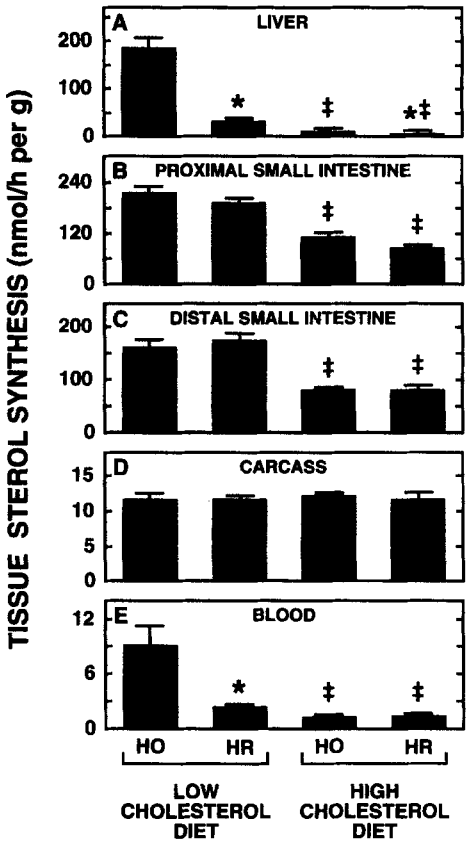

Fig. 6. Rates of cholesterol synthesis in the liver, small intestine, and remaining carcass measured in vivo in HO and HR monkeys fed either the low or high cholesterol diet for 5 months. The plasma lipids at the time of this study are summarized in Table 1. Each animal was given an intravenous bolus of [^{3}H]water and killed 1 h later. The liver, proximal and distal small intestine, and the entire remaining carcass were then saponified and the newly synthesized sterols were isolated. This figure shows the nmol of [^{3}H]water incorporated into newly synthesized sterols per h per g of tissue. Each value represents the mean ± 1 SEM for data obtained in the number of animals given in Table 1. *$P < 0.05$ for comparison with HO animals fed the same diet. ‡$P < 0.05$ for comparison with animals of the same phenotype on the low cholesterol diet.

kg bw, each animal was given a bolus of [^{3}H]water and killed 1 h later. **Figure 6** illustrates the in vivo rates of cholesterol synthesis, per g of tissue, in the three major tissue compartments of these two experimental groups. As reported before, the highest rate of sterol synthesis in this primate is found in the proximal small intestine (B) (3). However, the most important observation was that hepatic sterol synthesis in the HR animals occurred at only 16% (31 ± 4 nmol/h per g) of the rate seen in the HO animals (188 ± 42 nmol/h per g) (A). There were no significant differences seen in these two groups in the proximal or distal small intestine (B, C) or remaining tissues of the carcass (D). To be certain that this striking difference in hepatic synthesis was not the result of a genetically determined, excessive secretion of newly synthesized cholesterol out of the liver of the HR animals, aliquots of the liver and small intestine from these same animals were sliced and incubated in vitro with [1-^{14}C]octanoate. As shown in **Fig. 7,** the same relative differences in sterol synthesis in the various tissues that were demonstrated in vivo were also seen in these in vitro assays. Thus, the 6-fold higher rate of [^{3}H]water incorporation into sterols found in vivo in the liver of the HO animals (Fig. 6) reflected an actual difference in the rate of cholesterol synthesis in the liver that clearly differentiated these two phenotypes.

From the weights of these three groups of organs and the rates of sterol synthesis in each tissue (Fig. 6), the amount of cholesterol synthesis occurring in vivo in the three compartments and in the whole animal could be

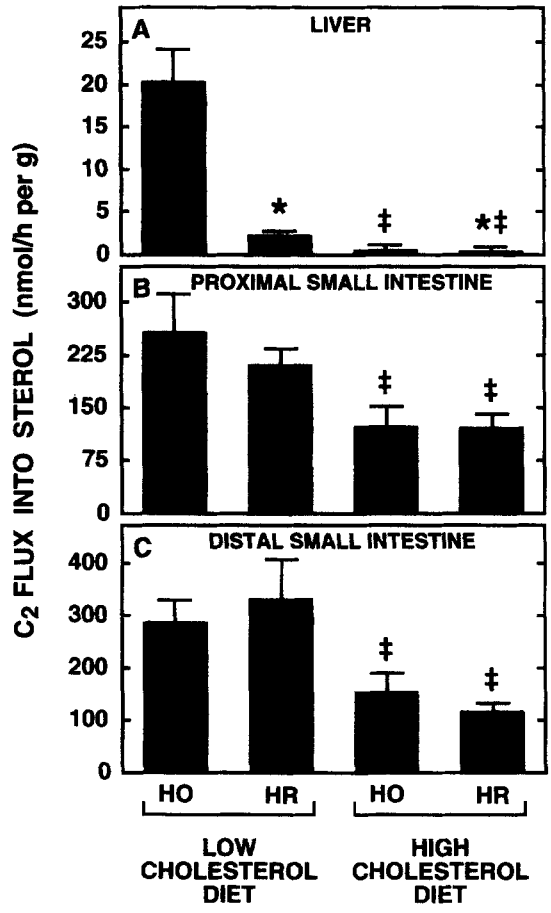

Fig. 7. Rates of cholesterol synthesis in the liver and proximal and distal small intestine measured in vitro. Aliquots of the liver and small intestine from the experiment described in Fig. 6 were sliced and incubated in vitro with [1-^{14}C]octanoate. This figure shows the rate of cholesterol synthesis expressed as acetyl CoA units incorporated into sterol per h per g of tissue. Each value represents the mean ± 1 SEM of data obtained in the number of animals given in Table 1. *$P < 0.05$ for comparison with HO animals fed the same diet. ‡$P < 0.05$ for comparison with animals of the same phenotype on the low cholesterol diet.

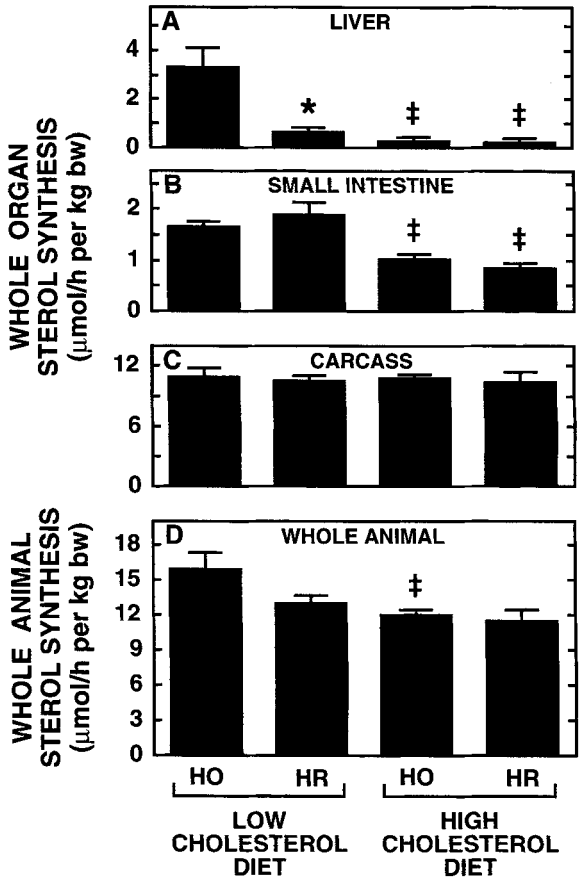

Fig. 8. Rates of cholesterol synthesis in the whole liver, small intestine, and remaining tissues of the carcass in vivo. The data in panels A, B, and C were calculated from the rates of synthesis shown in Fig. 6 and the appropriate whole organ weights, and these rates were then adjusted to a constant body weight of 1 kg. The content of newly synthesized sterols in the whole blood compartment were included with those of the liver (3). The rates of cholesterol synthesis in these three groups of tissue were added together to give the rates of whole animal sterol synthesis shown in panel D. Each value represents the mean ± 1 SEM of data obtained in the same number of animals given in Table 1. *$P < 0.05$ for comparison with HO animals fed the same diet. ‡$P < 0.05$ for comparison with animals of the same phenotype on the low cholesterol diet.

determined. As shown in **Fig. 8,** the great majority of synthesis in both groups occurred in the tissues of the carcass and equalled ~11 μmol/h per kg bw (C). In these animals fed the low cholesterol diet, hepatic synthesis was 3.3 ± 0.8 μmol/h per kg bw in the HO monkeys (20.2 ± 3.2% of whole animal synthesis), but only 0.6 ± 0.1 μmol/h per kg bw in the HR animals (only 4.9 ± 0.6% of whole animal synthesis) (A).

Effect of cholesterol challenge in the HO and HR phenotypes

While these measurements identified a major metabolic difference in the HO and HR animals, a final study was undertaken to examine these same parameters of cholesterol metabolism when the two phenotypes were challenged with a very large amount of dietary cholesterol. As also summarized in Table 1, when fed the high cholesterol diet for 5 months, the HR animals reached much higher concentrations of TC (499 mg/dl) (B) and LDL-C (412 mg/dl) (D) than did the HO animals (243 and 188 mg/dl, respectively). Furthermore, in response to this cholesterol challenge, the pool of hepatic cholesteryl esters in the HR monkeys (9.4 ± 2.1 mg/g) reached values nearly 3-fold higher than those achieved in the HO animals (3.9 ± 0.6 mg/g).

Under these conditions, the rate of cholesterol synthesis was markedly suppressed in the liver of both the HO and HR animals (Figs. 6A and 7A) and, further, this suppression was significantly greater in the HR (6 ± 1 nmol/h per g) than in the HO (11 ± 1 nmol/h per g) monkeys (Fig. 6A). Of particular note, this marked cholesterol challenge of 54 mg/day per kg bw also resulted in approximately a 50% suppression of both proximal and distal small intestinal cholesterol synthesis (Fig. 6B, C), but, importantly, no inhibition at all of the organs in the carcass (Figs. 6D and 8C). As the liver accounted for such a small percentage of whole animal synthesis and as cholesterol feeding did not suppress synthesis in the extrahepatic, extraintestinal organs, there were only relatively small differences in whole animal synthesis rates between the HO and HR animals or after cholesterol feeding (Fig. 8D). Thus, measurement of whole animal cholesterol synthesis by external sterol balance techniques would not have detected the critical difference in hepatic synthesis seen in the HO and HR animals and would have barely, if at all, detected the effects of cholesterol feeding. Such findings have been routinely reported in human studies where plasma cholesterol levels and the effect of cholesterol feeding are usually not reflected by changes in external sterol balance (43, 44).

Figure 9 summarizes the most important finding in this cholesterol challenge study. In the animals fed the low cholesterol diet, the level of cholesteryl esters in the liver was essentially the same, but the HO and HR phenotypes could be distinguished by the 6-fold difference in hepatic sterol synthesis (A, B). When challenged with a large amount of dietary sterol, the HO animal responded primarily by reducing the rate of cholesterol synthesis in the liver and, to a lesser degree, in the small intestine. The pool of excess sterol in the liver (and presumably in the regulatory pool) expanded only a small amount and brought about the modest increase in circulating LDL-C concentrations (Table 1). In contrast, the HR animal was much less able to compensate for the increased flow of cholesterol into the liver by suppression of synthesis so that the pool of excess cholesterol in the hepatocyte was markedly expanded (B), and

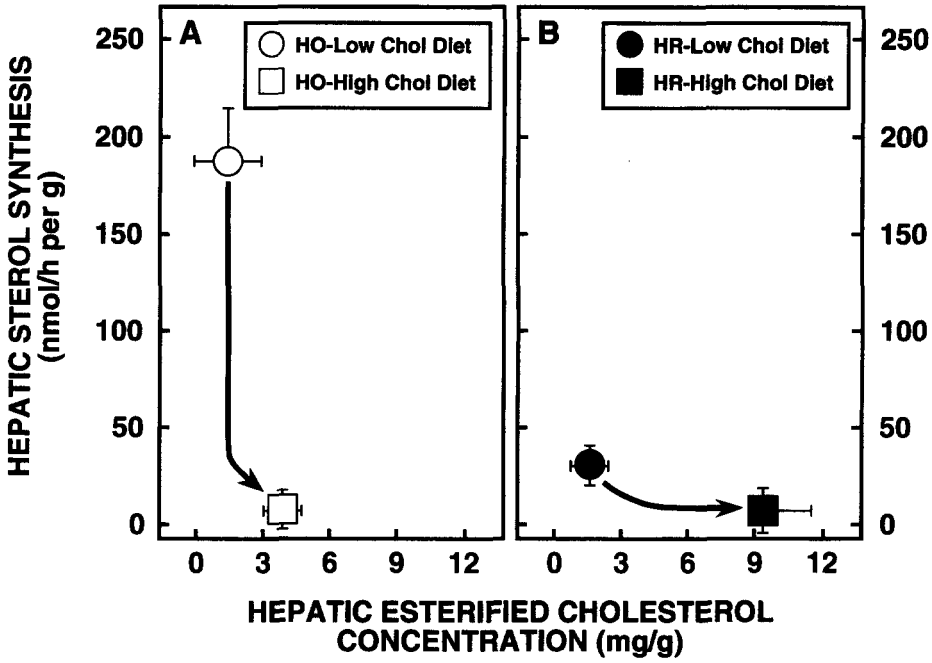

Fig. 9. Comparison of the rates of cholesterol synthesis and cholesteryl ester content in the liver of the HO and HR animals challenged with the high cholesterol diet. Each value represents the mean ± 1 SEM for the number of animals given in Table 1.

the plasma LDL-C concentration was elevated to a greater extent. Thus, after cholesterol challenge, the HO and HR phenotypes could be readily distinguished by the significant differences in the level of cholesteryl esters within the liver cells.

DISCUSSION

These studies demonstrated that in the steady state the HO and HR phenotypes could be clearly distinguished by a difference in net cholesterol balance across the liver under circumstances where dietary cholesterol intake was relatively low and constant over 5 months. When challenged with additional amounts of sterol, therefore, there was a disproportionate expansion of the cholesteryl ester pool in the liver and a more marked elevation of the circulating LDL-C concentration in the hyperresponsive animals. The detailed measurements of sterol balance carried out in these studies provide the basis for describing, in general terms, how this primate species responds to dietary cholesterol challenge and for explaining, in more quantitative terms, why there is a difference in the response of the plasma LDL-C concentration in the HO and HR phenotypes to this challenge.

The critical observations were carried out in animals maintained on a dietary cholesterol intake of 5.4 mg/day per kg bw, an amount that is comparable to the sterol intake of many humans (~380 mg/day per 70 kg individual). Earlier studies in this monkey species have shown that under these conditions about 2.9 mg/day per kg bw of the dietary cholesterol is absorbed (54%) while an additional 10.8 mg/day per kg bw is synthesized within the various organs of the body (3). The majority of this de novo synthesis occurs in the extrahepatic tissues (9.6 mg/day per kg bw) while only 1.2 mg/day per kg bw takes place in the liver (11% of whole animal synthesis). Such animals have a hepatic cholesteryl ester concentration of about 2.0 mg/g and an LDL-C production rate of about 16.5 mg/day per kg bw. Eighty percent of this lipoprotein fraction is cleared into the liver while LDL-C concentrations are in the range of 40–70 mg/dl (3). All of these values for cholesterol metabolism and LDL-C turnover, therefore, are very similar to those rates observed in young human subjects fed similar quantities of dietary sterol (2, 44–46).

The general responses of this primate to increased dietary cholesterol intake are well illustrated by these studies. When individual animals were challenged with increased daily amounts of sterol over the 5-month experimental period, there was no expansion of the pool of excess cholesterol in the hepatocytes until hepatic synthesis had been suppressed nearly to zero (Fig. 3).

Beyond this point, however, further increases in the intake of dietary sterol were associated with expansion of the intracellular cholesterol concentrations, and nearly all of this excess sterol was found in the ester pool (Fig. 4). At the highest levels of dietary cholesterol challenge (54 mg/day per kg bw) there was nearly complete inhibition of sterol synthesis in the liver and partial suppression in the proximal and distal small intestine. There was, however, no significant change in the rate of cholesterogenesis in the tissues that comprise the carcass (Figs. 6 and 8). These findings are consistent with earlier observations in other species that cholesterol carried in lipoproteins of intestinal origin are, for practical purposes, taken up only by the liver. In response to dietary cholesterol challenge, therefore, of all the extraintestinal organs only the liver manifests suppression of sterol synthesis and expansion of the intracellular pool of cholesterol (47, 48).

This expansion of the cholesteryl ester pool, in turn, is presumably associated with partial suppression of hepatic LDLR activity and an increase in the LDL-C production rate that, together, account for the increases in steady-state LDL-C concentrations observed in these monkeys (Table 1). This does not imply, however, that it is the sterol ester fraction in the liver that regulates these events. Rather, under conditions where dietary triacylglycerol intake and the profile of fatty acids in the liver is constant (Fig. 2), increasing loads of dietary cholesterol apparently expand in parallel the regulatory pool of unesterified sterol in the endoplasmic reticulum (49, 50) and the storage pool of cholesteryl esters. As a result, the level of hepatic LDLR activity (J^m) varies inversely, and the LDL-C production rate (J_t) varies directly, with the steady-state concentration of cholesteryl esters (20, 21, 40, 51). In effect, under the conditions utilized in these experiments, the level of hepatic cholesteryl esters becomes a surrogate marker for the size of the regulatory pool.

Beyond this general description of how the cynomolgus monkey responds to dietary sterol challenge, however, these studies provide quantitative data on cholesterol balance across the major organ systems of this animal and illuminate the differences in this balance that exist in the HO and HR phenotypes. These differences are best appreciated by converting the rates of [^{3}H]water incorporation into sterols (Fig. 8) to absolute rates expressed as the mg of cholesterol synthesized per day per kg bw. As summarized in **Fig. 10,** these rates of synthesis are shown for the HO (number within circle) and HR (number adjacent to circle) animals under conditions where dietary triacylglycerol and fiber were constant and where dietary cholesterol intake equalled 5.4 mg/day per kg bw. Under these circumstances the rates of cholesterol synthesis in the small intestine (1.2 versus 1.4 mg/day per kg bw) and extrahepatic tissues (8.2 versus 7.8 mg/day per kg bw) were not significantly different in the two phenotypes (Fig. 8). In contrast, synthesis in the liver of the HR animals equalled only 0.4 mg/day per kg bw whereas in the HO animals this rate was 6-fold higher and equalled 2.5 mg/day per kg bw. Even though this difference in hepatic synthesis was easily detectable, cholesterol balance across the whole animal was not significantly different and equalled 17.3 and 15.0 mg/day per kg bw, respectively, in the HO and HR monkeys (Fig. 10). Furthermore, despite this difference in the rate of synthesis in the liver, there were no differences in the concentration of hepatic cholesteryl esters (Fig. 9) or in the concentration of LDL-C in the blood (Table 1) at this level of dietary cholesterol intake.

Nevertheless, it was clear from these measurements that the HR animals had a net excess in sterol balance across the liver equal to 2.1 mg/day per kg bw compared to the HO monkeys. There are only three pathways where polymorphisms in critical enzymes or transporters could account for this difference in cholesterol balance across the liver. The most important of these sources for a net contribution of sterol to the liver is de novo synthesis in the extrahepatic tissues (~ 8 mg/day per kg bw, Fig. 10); however, these studies revealed no difference in this rate in the HO and HR monkeys (Fig. 8). Alternatively, the HR animals must have received an additional 2.1 mg/day per kg bw from the intestine or excreted 2.1 mg/day per kg less sterol from the liver as either cholesterol or bile acids (Fig. 10). The subtlety of these differences should be emphasized. As the dietary intake of cholesterol in these monkeys equalled 5.4 mg/day per kg bw, it would only be necessary, for example, that the HO monkey absorbed ~ 25% of this dietary load while the HR animals absorbed ~ 60%. Such variability in net cholesterol absorption (or net hepatic sterol excretion) is well within the range reported in both the monkey and human (31, 52, 53). Indeed, these theoretical values are remarkably close to those actually found in two studies of hyper- and hyporesponder African green and rhesus monkeys (27, 31). Increasing the dietary cholesterol intake by only an additional 1–2 mg/day per kg bw (70–140 mg/day per 70 kg human) would be sufficient to begin to expand the regulatory and ester pools of sterol in the liver (Fig. 9) and raise the plasma LDL-C concentration in the HR, but not in the HO, animals.

Thus, these studies indicate that the HO and HR phenotypes are due to a difference in net sterol balance across the liver that is attributable to events occurring during either intestinal cholesterol absorption or net sterol excretion from the liver. While future studies will have to distinguish which of these two possibilities ac-

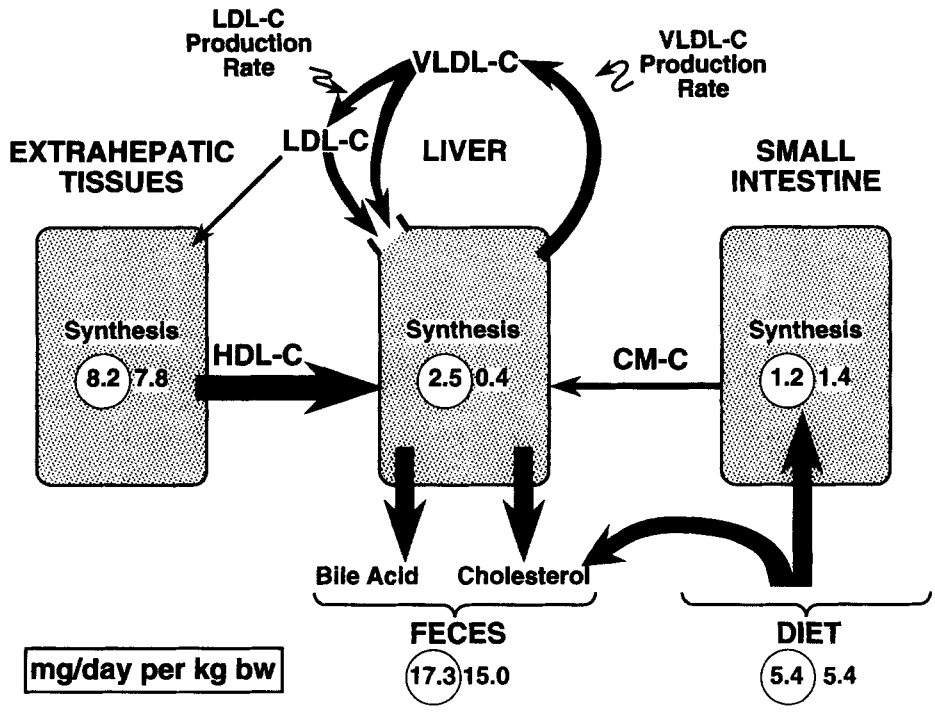

Fig. 10. Net cholesterol balance across the major tissues of the HO and HR monkeys fed 5.4 mg of cholesterol per day per kg bw for 5 months. Using the set of data for cholesterol synthesis, the flux of sterol through the various tissue compartments of the HO (numbers within the circles) and HR (numbers adjacent to the circles) monkeys were calculated. Each of these numbers represents mg of cholesterol per day per kg bw.

count for this difference in cholesterol balance across the liver, these investigations focus attention on a relatively few specific proteins where polymorphisms in genes could account for these two phenotypes. On the one hand, these genetic effects could be exerted through the proteins that dictate the magnitude of intestinal cholesterol absorption by altering intestinal unstirred layer resistance, microvillus permeability or sterol esterification (42, 54–56). On the other hand, this difference might be the manifestation of polymorphisms in the proteins that dictate net sterol excretion from the liver through alterations in canalicular cholesterol transport, cholesterol 7α-hydroxylase or oxysterol 7α-hydroxylase activity, or ileal bile acid absorption (57–61). Which one, or more, of these genetic polymorphisms account for these two phenotypes remains to be elucidated.

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