Cholesterol absorption in baboons

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Abstract We estimated the effects of age, sex, saturated and polyunsaturated dietary fat, and two levels of dietary cholesterol intake on cholesterol absorption in juvenile baboons between 15 and 51 months of age. We compared the reproducibility of methods of measuring cholesterol absorption within animals, and also compared estimates of percentage cholesterol absorption by three different methods. Cholesterol absorption was significantly greater in all diet groups at 27 and 39 months of age than it was at 15 or 51 months ($P < 0.001$). Cholesterol absorption (%) was significantly greater in animals fed low cholesterol diets than it was in animals fed high cholesterol diets. However, cholesterol absorption was not affected by type of fat (saturated or unsaturated) in the diet or by sex. The range of reproducibility (within-animal standard deviation) of percentage cholesterol absorption for different diet groups was 3.35 to 3.90% for the Borgström method (1969. *J. Lipid Res.* **10:** 331–337), 4.67 to 7.30% for the Sodhi method (1971. *Proc. Soc. Exp. Biol. Med.* **137:** 277–279), and 3.69 to 7.15% by a combined isotopic chromatographic method (Method I). Percentage absorption measured by either the Sodhi method or Method I was significantly greater than that measured by the Borgström method.—**Mott, G. E., E. M. Jackson, and M. D. Morris.** Cholesterol absorption in baboons. *J. Lipid Res.* 1980. **21:** 635–641.

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Interest in the role of dietary cholesterol in regulation of serum cholesterol concentration and cholesterol metabolism has stimulated the development of a number of methods of measuring cholesterol absorption (1–6). When these methods have been used to determine the relationships between dietary factors or certain aspects of cholesterol metabolism and cholesterol absorption the results have often been conflicting.

A difficulty in investigating these metabolic relationships is the large methodologic and biologic variability associated with cholesterol absorption in primates. To explain the more complex relationships in cholesterol metabolism, an estimation of the magnitude of the methodologic and biologic variability and the effects of dietary factors on cholesterol absorption is essential. Therefore, to establish the sources of variability, we estimated the reproducibility (within-animal standard deviation) of cholesterol absorption measurements by several methods and measured the effects of age, sex, dietary cholesterol, and type of fat on percent cholesterol absorption in baboons. The results provide a basis for designing experiments in which cholesterol absorption is measured.

MATERIALS AND METHODS

Animals and diets

The animal subjects were from a group of 97 baboons (*Papio cynocephalus*) born at the Southwest Foundation for Research and Education (SFRE) and weaned at 3 months of age.

The diets fed after weaning had a base of Special Monkey Chow 25 (#5045-6) (Ralston Purina Co., St. Louis, MO) to which different fat mixtures, USP grade cholesterol (Vitamins Assoc., Chicago, IL) and vitamins A and C were added. The fat mixtures, derived from safflower, palm, cottonseed, and soybean oils, were supplied by Dr. Fred Mattson of the Proctor and Gamble Co., Cincinnati, OH. The saturated fat had a polyunsaturated to saturated fatty acid ratio (P/S) of 0.26 and the polyunsaturated fat had a P/S of 2.14. **Table 1** shows the final fat and cholesterol contents and the fatty acid composition of the diets. Each batch of diet fed during the absorption studies was analyzed for cholesterol and β-sitosterol content. The contribution of the various dietary components to the total caloric content of the diets was: carbohydrate, 38.0%; protein, 22.1%; and fat, 39.9%. Immediately before each feeding, feces were collected from the collection pans and the pans were cleaned and dried. Two hundred-gram portions of the assigned diet were made available to each animal for 30 min twice a day and the uneaten food then was collected from the cage pans and weighed.

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TABLE 1. Diet sterol and fatty acid content

A. Cholesterol and β-sitosterol content of diets

Diet	Diet Code	Cholesterol	β-sitosterol
		mg/g diet	
Low cholesterol-unsaturated fat	LC-UF	0.043	0.817
Low cholesterol-saturated fat	LC-SF	0.032	0.557
High cholesterol-unsaturated fat	HC-UF	3.411	0.800
High cholesterol-saturated fat	HC-SF	3.370	0.559

B. Fatty acid composition of added fats

Fatty Acid	Type of Dietary Fat	
	Saturated (SF)	Unsaturated (UF)
	% of total	
14:0	0.7	0.6
16:0	35.7	17.0
18:0	8.4	4.6
18:1	43.8	30.4
18:2	11.5	47.5

Reagents

The radioactive sterols [1α,2α(n)-^{3}H]cholesterol, and [4-^{14}C]β-sitosterol were purchased from Amersham Corp., Arlington Heights, IL or from New England Nuclear, Boston, MA and were purified by thin-layer chromatography on Silica Gel 60 (Cat. #5763, EM Reagents, Elmsford, NY) in a system of diethyl ether–hexane 70:30 (v/v). Steroid standards and gas–liquid chromatography supplies were purchased from Applied Science Laboratories (State College, PA).

Radioactivity measurements

Radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb Model 3380 liquid scintillation counter. DPM's were calculated from quenched standard curves by an external standard channels ratio technique.

Analysis of neutral steroids of feces or feed

The feces were collected daily in ½ gal Mason jars and were stored at −20°C until analyzed. The specimens were thawed and homogenized with water (1:1) in the collection jar on an Oster blender. Duplicate (~2g) aliquots were weighed to the nearest mg, saponified, and extracted with petroleum ether (7). Aliquots of the extract were taken for counting radioactivity or for quantitating the neutral steroids by gas–liquid chromatography. The sterols were analyzed as trimethylsilyl ethers on a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector and an automatic sampler. Samples were injected on a 1.5 m × 4 mm I.D. glass column packed with 3% OV-17 at 240°C.

The sterol content of feed was determined by the same procedure without the initial homogenization step.

Cholesterol absorption methods

Borgström method (1, 2). A dose of 10 μCi [1,2-^{3}H]-cholesterol and 5 μCi [4-^{14}C]β-sitosterol dissolved in 100 μl acetone was added to the center of a 7-g ball of the animal's diet and was fed to the animal before its usual 200-g meal. Feces were collected for 6 days and aliquots of the total 6-day fecal collections were analyzed in duplicate for radioactivity as described above.

Sodhi method (3). In the Sodhi method, a modification of the Borgström method, the isotope ratio of cholesterol and β-sitosterol is determined from a single stool specimen rather than from an aliquot of a pooled 5–6 day fecal collection (8). The radioactive sterols were given to the animals for the Borgström absorption procedure with a feed-ball which also contained 60–100 mg Carmine (alum lake) as a fecal marker. The fecal sample for analysis was taken from the feces containing the most marker and analyzed for radioactivity as described above. Cholesterol absorption was calculated as described by Sodhi et al. (3).

Isotopic chromatographic method (Method I) (2, 9). Intravenous doses of about 30 μCi of [4-^{14}C]cholesterol per animal were administered in baboon serum according to the procedure of Goodman and Noble (10). The amount injected was determined by counting a weighed sample, weighing the solution in the syringe, and correcting for the residual radioactivity in the syringe and catheter after injection. Twenty-five blood specimens were drawn at different intervals during the 4 months following injection and the specific radioactivity of serum cholesterol was determined. Serum cholesterol was measured enzymatically (11) with an ABA-100 instrument (Abott Diagnostics, S. Pasadena, CA). Serum radioactivity was determined by adding 0.5 ml of whole serum directly to 10 ml of Scintisol (Isolabs Inc., Akron, OH) and counting by liquid scintillation spectrometry. Separate 5-day fecal collections were made at 2 months and 3 months after injection of the radioactivity and fecal aliquots were saponified and extracted as described above. The mass of endogenous[4] neutral steroids was determined by dividing the total counts in the neutral steroid fraction

[4] As used here, "endogenous" refers to that component of the fecal steroids that was in equilibrium with plasma cholesterol, i.e., that derived from bile or that fraction of cholesterol derived from the intestinal mucosa that is in equilibrium with plasma.

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excreted during the 5-day fecal collection by the serum cholesterol specific radioactivity measured 1 day before the mid point of the fecal collection. Also, the total mass of neutral steroids in the feces was determined by the gas–liquid chromatographic method described above. Cholesterol absorption could not be estimated by Method I for the two low cholesterol diet groups because the difference between the excretion of endogenous neutral steroids and the mass of neutral steroids determined by gas–liquid chromatography was much greater than the daily cholesterol intake. Cholesterol absorption was calculated for the high cholesterol groups as described by Grundy and Ahrens (9) (Equations 4, 10, and 11).

Correcting percentage cholesterol absorption for ^3H losses

In the course of these absorption experiments we observed low counts of tritium which could not be extracted with petroleum ether from the saponified sample. Generally, less than 5% of the ^3H counts were lost, but in a few samples this loss was 10–15%. We recently reported (12) that when intestinal bacteria convert [1,2-^3H]cholesterol to [1,2-^3H]coprostanone, approximately one-half of the tritium can be lost by enolization of ^3H from the 2 position of [1,2-^3H]-coprostanone. This loss would result in significant overestimation of the percentage cholesterol absorption by methods requiring fecal radioactivity recovery. The data in the present paper obtained with [1,2-^3H]-cholesterol and [4-^{14}C]β-sitosterol were corrected for ^3H loss from coprostanone as we described. This correction was a mean of 2.3% cholesterol absorbed for the animals ingesting low cholesterol diets and 5.2% for animals ingesting high cholesterol diets.

Statistical methods

The effects of level of dietary cholesterol, type of dietary fat, sex, and age on the Borgström method measurements of percent cholesterol absorption (Table 2) were investigated in a repeated measures analysis of variance (13). The sex effect and its interactions were not significant and were not included in the model used in the final analysis.

TABLE 2. Mean percentage cholesterol absorption measured by the Borgström method according to age and diet groups

Diet	Number of Animals	Age (months) 15	27	39	51
		mean % (±SD)			
LC-UF	14	47.8 (13.3)	55.0 (9.4)	56.5 (6.9)	47.4 (6.5)
LC-SF	14	46.6 (7.6)	52.3 (7.5)	53.0 (6.0)	49.7 (5.2)
HC-UF	16	33.9 (10.6)	36.8 (6.1)	37.4 (9.8)	31.1 (5.2)
HC-SF	18	28.9 (10.2)	39.5 (9.1)	35.5 (4.8)	32.9 (4.9)

TABLE 3. Reproducibility (within-animal standard deviation) of three methods of measuring percentage cholesterol absorption in juvenile baboons

Diet	Method: Borgström	Sodhi	Method I
	within-animal SD (No. of animals)		
LC-UF	3.39 (10)	5.48 (11)	
LC-SF	3.55 (14)	7.30 (12)	
HC-UF	3.35 (14)	5.05 (14)	3.69 (22)
HC-SF	3.90 (15)	4.67 (12)	7.15 (23)

The within-animal standard deviations, as measures of reproducibility, were calculated by one way analysis of variance for each method-diet combination (Table 3). Comparisons were made among the methods, within diet groups, using F statistics.

Methods also were compared for systematic differences. Within diet groups, paired-sample t tests (14) were performed, using data from animals 3 years of age for which two or three methods of determining cholesterol absorption had been employed. Means and standard deviations for percent cholesterol absorption also were computed (Table 4) for each method-diet combination.

RESULTS

Age and diet effects

The mean percent absorption of dietary cholesterol measured annually from 15–51 months of age by the Borgström method is shown in **Table 2.** There was a significant effect of age on absorption ($P < 0.001$). The percent cholesterol absorbed was higher in all diet groups at 2 and 3 years of age than either in infancy or after puberty. The percent cholesterol absorption in the high cholesterol diet groups was significantly lower than in the low cholesterol groups ($P < 0.001$). The type of fat had no significant effect on absorption and no significant diet by age interactions were found.

Reproducibility of absorption measurements

The reproducibility of each of the three methods was estimated by repeating the absorption measurements twice in each animal. **Table 3** shows small and very similar within-animal standard deviations among the diet groups for the Borgström method. Compared to the Borgström method, the reproducibility of the Sodhi method was not as good (higher within-animal SD) and was more variable among the diet groups.

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TABLE 4. Mean percentage cholesterol absorption and overall standard deviation by three methods in 39-month old baboons in four diet groups

Diet	Number of Animals	Method		
		Borgström	Sodhi	Method I
		mean % (±SD)		
LC-UF	20	53.6 (6.7)	61.0 (7.6)	
LC-SF	14	51.5 (5.7)	58.4 (6.3)	
HC-UF	11	33.7 (6.4)	38.3 (8.0)	43.8 (9.3)
HC-SF	10	37.0 (5.3)	41.3 (4.5)	46.3 (7.0)

However, only the within-animal standard deviation of the LC-SF group was significantly lower by the Borgström method compared to the Sodhi ($P < 0.01$) and the within-animal standard deviation of only the HC-SF group was significantly lower by the Borgström method compared to Method I ($P < 0.01$). There were no significant differences between the Sodhi method and Method I.

Percent cholesterol absorption by three methods

Table 4 shows that absorption measured by Method I is significantly higher than by the Borgström method for both of the high cholesterol diet groups ($P < 0.001$). The differences between Method I and the Sodhi method are not significant. The absorption values by the Sodhi method are significantly ($P < 0.001$) higher than the values obtained by the Borgström method for all four diet groups at 39 months of age and this was true also at 51 months of age (data not shown).

Daily excretion of labeled sterols

The daily percent of the total 6-day excretion in feces of oral doses of [^{3}H]cholesterol and [^{14}C]β-sitosterol from baboons at 4 years of age is shown in **Table 5.** The values were obtained by dividing the daily recovery of cholesterol or β-sitosterol by the total 6-day recovery of that sterol and multiplying by 100. A difference in retention of the two sterols is indicated by recovery during day 2 of a greater percentage of the total excreted β-sitosterol than cholesterol. However, from days 4–6 the proportion of radioactive cholesterol was greater than β-sitosterol. The differential rate of excretion of the two sterols is reflected in the difference in absorption values determined by the Sodhi and Borgström methods (Table 4). β-Sitosterol also is retained to some extent, i.e., as much as 4% was excreted 2 days after excretion of the fecal flow marker on day 2. Recovery of labeled β-sitosterol averaged 69–83% in the various experiments.

DISCUSSION

Age and diet effects

The increase in cholesterol absorption during the second and third years was consistent for all diet groups. This period is immediately prior to the onset of puberty when large changes in hormonal patterns occur. Inspection of growth curves for 16 males and 16 females from these groups did not indicate a possible relationship between change in body weight and percentage cholesterol absorption. At 51 months

TABLE 5. Daily percent of total 6-day excretion in feces of oral doses of [^{3}H]cholesterol and [^{14}C]β-sitosterol from baboons at 4 years of age

Animal/Diet	Sterol	Day					
		1	2	3	4	5	6
		%[a]					
X-301/LC/SF	Cholesterol	0.36	67.8	19.3	7.63	3.93	1.03
	β-sitosterol	0.05	75.7	18.8	4.00	1.15	0.25
X-282/LC-SF	Cholesterol	2.53	55.8	26.4	8.36	4.02	2.93
	β-sitosterol	4.35	66.3	24.4	2.39	2.06	0.26
X-238/HC-UF	Cholesterol	0.03	58.8	32.3	5.38	3.12	0.43
	β-sitosterol	0.02	61.8	32.0	4.33	1.68	0.13
X-403/HC-SF	Cholesterol	0	32.7	54.8	7.36	3.96	1.14
	β-sitosterol	0.01	36.4	56.9	4.67	0.39	1.56
X-396/HC-SF	Cholesterol	0	17.1	55.7	22.9	3.07	1.25
	β-sitosterol	0	19.0	58.8	21.4	0.72	0.09
X-383/HC-SF	Cholesterol	0.07	36.5	47.4	10.7	2.90	2.52
	β-sitosterol	0	40.4	50.3	7.9	0.82	0.47

[a] Percent of radioactive sterol recovered in total of six daily samples.

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of age, the percentage absorption returned to approximately the 15 month level.

Early studies of the effects of type of fat on cholesterol absorption produced conflicting results (15–17). More recent results obtained with improved methods also suggest large individual variations and variable effects of different types of fats. Grundy and Ahrens (18) found that polyunsaturated fat enhanced cholesterol absorption in two of five patients ingesting formulas containing safflower oil or corn oil compared to butter oil. Other investigators also observed higher cholesterol absorption in squirrel monkeys (19) and rats (20) ingesting diets containing safflower oil compared to butter oil. However, in three species of monkeys, no difference in cholesterol absorption was observed between animals fed either of the unsaturated oils, safflower or corn oil, and the more saturated oil, coconut oil (21). In the present experiments we did not find significant differences in cholesterol absorption between young baboons fed saturated or unsaturated fats, both of plant origin.

The plant oils also contain the plant sterols, β-sitosterol, campesterol, and stigmasterol, which interfere with cholesterol absorption. However, the level of plant sterol intake by these animals, approximately 400 mg/day for an animal fed unsaturated fat and 300 mg/day for those fed saturated fat, would be expected to have a minimal effect on cholesterol absorption (22).

Reproducibility of absorption measurements

Quintão, Grundy, and Ahrens (2) determined the reproducibility of cholesterol absorption with the Borgström method 3–5 times in each of six hypercholesterolemic patients who had a cholesterol intake from 0.1 to 1.0 mg/Kcal of diet. The pooled SD calculated from these repeated measures was approximately 4.8% which was similar to the within-animal variability, SD ≅ 3–4% (Table 3), that we observed in this study. The reproducibility (within-animal SD) (Table 3) of the Borgström method was 3.35–3.90% absorption or a coefficient of variation of 7–12%. The higher variability of the Sodhi method (within-animal SD = 5.05–7.30%) is probably due to differences in sampling time. For the Sodhi determination, the sample was obtained when the fecal flow marker administered with the labeled sterols was excreted 8–30 hr later. However, as shown in Table 5, the $^{3}H/^{14}C$ of cholesterol and β-sitosterol excreted is changing rapidly during this time and therefore is subject to large sampling errors due to time. A similar difference in intestinal transit of cholesterol and β-sitosterol was observed in the rat by Sodhi et al. (8).

In another study (18), the reproducibility of absorption estimates in five patients was generally low and quite variable by Method I. Within individuals, standard deviations of the percent absorbed ranged from approximately 7 to 22% on two diet regimens. We calculated a pooled SD of approximately 10.4% from the results of Sedaghat et al. (23) using Method I, in which several fecal collections were made in three hyperlipidemic patients after a single injection of radioactive cholesterol. The reproducibility in baboons of Method I varied widely between the two diet groups (within-animal SD = 3.69 and 7.15%, Table 3).

In one of the few reports of within-animal variability of cholesterol absorption in nonhuman primates, Tanaka and Portman (19) performed two tests on each of five squirrel monkeys by the Zilversmit method (24). The low cholesterol, high fat diets resulted in percent cholesterol absorption in the range 45–78%. The within-animal standard deviation we calculated from the data for these five animals was 3.24%.

The reproducibility of a method or the within-animal variability has two components: the methodologic variability and the biologic variability due to absorption differences over time. Presumably the latter component becomes smaller the closer in time the repeated measurements are made, although Grundy and Mok (5) suggest that cholesterol absorption varies considerably within hours or even minutes throughout the day. From a practical standpoint the measurements can be repeated only every 2–4 weeks, except by Method I or by several recent methods (5, 6).

Fecal collections may be a larger source of methodologic error in nonhuman primates. Although theoretically measurements of [^{14}C]β-sitosterol excretion could be used to correct for fecal losses, Table 5 indicates an unequal rate of excretion of β-sitosterol and cholesterol. This difference may introduce errors of several percent in the percent absorbed by the Borgström method if fecal losses occur during the 1 or 2 days of maximum excretion of radioactivity. Also this difference would produce larger errors in the Sodhi method as described above. An analysis of the mass of dietary β-sitosterol recovered in the feces should overcome this problem, but only for the Borgström method. A large methodologic variability may be encountered with Method I since the calculations rely on several measurements which are based on a number of assumptions which are difficult to validate in individual animals. The Borgström method, therefore, appears to be the most reproducible of the three methods considered.

The error in analysis of fecal radioactivity is a small component of the variability as demonstrated by a

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SD of 1.25% (coefficient of variation = 2.7%) of the percent cholesterol absorbed by the Borgström method for duplicate analyses of 24 fecal samples.

Percent cholesterol absorption by three methods

The percent absorption measured by the Borgström method was consistently lower than that measured by the Sodhi method. Differences between the two methods also have been reported for rats and humans (8). The data in Table 5 explain these differences. The percentage of the total 6-day collection of radioactive cholesterol (and metabolites) excreted within 1 or 2 days of the dose is less than the percentage of β-sitosterol (and metabolites) excreted. Samples taken on those days, as is done for the Sodhi procedure, would result in an overestimate of the percent absorbed compared to data obtained from a 6-day fecal pool. This differential rate of excretion of cholesterol and β-sitosterol is apparently due to uptake of dietary cholesterol but not β-sitosterol by the intestinal wall. However, this bound cholesterol is not transported into the lymph and returns to the lumen. This process is described as "exchange" although the precise mechanism is unknown.

Feeding a diet similar to our HC-SF diet to adult baboons, Eggen (25) found a mean of 37% cholesterol absorption by Method I. This value was similar to our estimate for the HC-SF group by the Borgström method (Table 3), but nearly 10% lower than we obtained by Method I. In that study Eggen approximated feed intake by an indirect method which may have underestimated feed consumption and therefore resulted in a lower percent cholesterol absorption. The higher estimates of absorption we found by Method I compared to the Borgström method could result from either an overestimation of dietary cholesterol intake due to losses of the feed or an underestimation of the endogenous neutral steroids. The latter possibility is supported by our recent findings that baboons fed the low cholesterol diets (5–10 mg cholesterol intake/day) had fecal neutral steroid specific radioactivities that were 20–40% lower than the specific radioactivity of serum cholesterol.[5] These results indicate that cholesterol entering the intestinal tract from bile or the intestinal mucosa had not equilibrated with the plasma radioactive cholesterol. Therefore, we found it impossible to estimate cholesterol absorption by Method I in baboons fed low cholesterol diets. A similar lack of equilibration of serum radioactive cholesterol with endogenous cholesterol entering the intestinal tract of animals fed the high cholesterol diet would result in an overestimation of the amount absorbed by Method I.

The accuracy of these methods is not known. However, serious difficulties with both the Sodhi method and Method I suggest that the Borgström method may give the best estimate of true absorption values in baboons.

Practical considerations

The recovery of fecal radioactivity in nonhuman primates is difficult and requires special metabolic cages and meticulous care. The use of β-sitosterol or another recovery marker is necessary. This precaution is particularly important for animals fed high fat, low bulk diets from which stool collections are small and soft. However, it seems unlikely that poor fecal recovery can account for the β-sitosterol losses of 15–30% we encountered in baboons. Other investigators also have observed losses of 40–60% of dietary β-sitosterol in several species of monkeys (21, 26) and even greater losses in baboons (26). Those investigators speculated that the large losses of β-sitosterol could result from degradation by intestinal bacteria. We could not find support for this suggestion by comparing the fecal recovery of β-sitosterol with Cr_2O_3 in 70 baboons 15 months of age.[5] The mean recovery of β-sitosterol was 77.7% and of Cr_2O_3, 76.5%; results which indicated no significant degradation of neutral sterols.

Design of absorption experiments

Using the overall variability of absorption measurements within a diet group, the size of experimental groups required to detect a given percent difference in cholesterol absorption can be approximated (27). For example, with a standard deviation of percent cholesterol absorption of 5–7% as shown in Table 4, group sizes of ten animals would be required to detect a 10% difference in percent cholesterol absorption with a probability of 0.90 and $P = 0.05$. However, to detect a 5% difference under the same conditions, 33 animals per group would be required. The measures of reproducibility could be used in a similar manner to determine the number of absorption measurements required to detect absorption differences between two animals.

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[5] Mott, G. E. Unpublished observations.

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