Evaluation of an isotope ratio method for measuring biliary cholesterol secretion

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Abstract We have evaluated an isotope ratio method for measuring biliary cholesterol secretion. Secretion was measured in eight nonhuman primates by analysis of radioactivity of feces and plasma 4 weeks after intravenous administration of a single dose of [^{3}H]cholesterol. For the test, [^{14}C]cholesterol was fed in known amounts daily for 10 days. The ratio of isotopes in feces (^{14}C/^{3}H) was equivalent to the ratio of total radioactivity that entered the intestine from diet and bile. Assuming biliary cholesterol specific activity equals plasma cholesterol specific activity, the mass of cholesterol secreted daily in the bile could be calculated. In paired experiments in four animals we were able to directly compare biliary secretion by the new method with mass measurement by intraduodenal intubation (Grundy, S. M., and A. L. Metzger. 1972. *Gastroenterology*. **52**: 2100–1216). The two methods correlated well in these four animals ($r = 0.97$). We further noted that bile secretion by the new method (eight animals) and by the mass method (four animals) correlated well with body weight ($r = 0.94$ for weight vs secretion by the isotope ratio method; $r = 0.97$ for weight vs secretion by the mass ratio method). Ranges of body weight and secretion for the eight animals were 4.2–13.1 kg and 3.6–11.4 mg/hr, respectively. The slopes of the two regression lines for body weight vs. secretion measured by the two methods did not differ significantly from one another ($F_{(1,8)} = 1.42$; $0.25 < P < 0.50$). Cholesterol secretion was measured directly by bile duct cannulation in one animal. Secretion in this animal approximated that expected for its weight by the regression line of weight vs secretion measured by the isotope ratio method. This new method does not require intraduodenal intubation, provides sequential daily measurements over a period of 5 days, and requires only plasma and stool (aliquot) collection for isotope analysis.— Crouse, J. R., M. D. Wilson, and D. S. Weaver. Evaluation of an isotope ratio method for measuring biliary cholesterol secretion. *J. Lipid Res.* 1983. **24**: 854–860.

Supplementary key words nonhuman primates

In 1972 Grundy and Metzger (1) described a method for measuring biliary lipid secretion that relied on intraduodenal intubation and infusion of a fat-containing formula over 10 hr. This technique provided a means of gaining insight into the pathophysiology of cholesterol cholelithiasis (2) and the role played by cholesterol secretion in a number of clinical conditions such as obesity (3), fasting (4), polyunsaturated fat feeding (5), Atromid-S administration (6), oral contraceptive use (7), and chenodeoxycholate administration (8). The method also proved useful in defining differences in cholesterol secretion that differentiated Pima Indians from non-Indian volunteers (9).

In addition to this method's usefulness for measuring biliary secretion of cholesterol, when combined with a measure of percent cholesterol absorption, the method affords quantitation of the mass of cholesterol absorbed daily (10).

In 1978, we evaluated a new isotope ratio method for measuring cholesterol absorption that necessitated oral administration of isotopic cholesterol for 10 days (11). We found then that absorbed dietary isotope was not resecreted during the first 10 days of administration. This observation prompted us to evaluate a new isotope ratio method (IRM) for measuring biliary cholesterol secretion.

METHODS

General experimental design

Biliary secretion of cholesterol was measured in nine nonhuman primates. Animals ranged in weight from 4.2 to 13.1 kg (Table 1). Semi-solid diets were prepared from lactalbumin, wheat flour, applesauce, dextrose, sucrose, casein, vitamins, cholesterol, β-sitosterol, and safflower oil (diet A) or lard (diet B) (12, 13). Animals maintained their body weight throughout the experiment. All animals except #9 were injected with [1,2-^{3}H]cholesterol 6 weeks prior to the study for measurement of cholesterol secretion by the isotope ratio method (see below). Double-lumen indwelling intraduodenal tubes were positioned surgically in four animals (#2, 3, 4, and 6) for measurement of cholesterol secretion by the mass ratio method of Grundy and Metz-

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Abbreviations: IRM, isotope ratio method; TLC, thin-layer chromatography.

ger (1). After recovery from surgery (4–12 days), each animal underwent the first of two studies of cholesterol secretion by the mass ratio method (Mass 1, Table 1). The animals were fed [4-^{14}C]cholesterol daily for 10 days. Stool was collected daily and blood samples were obtained three times for measurement of cholesterol secretion by the isotope ratio method as described in detail below. Cholesterol secretion was then assessed a second time by the mass ratio technique (Mass 2, Table 1). In these four animals duplicate analyses of cholesterol secretion by the mass ratio method could be averaged and compared to analyses by the isotope method.

In four other animals (#1, 5, 7, and 8), cholesterol secretion was measured by the isotope ratio method only.

One African green monkey (#9) underwent bile duct cannulation and bile diversion. Details of the procedure are described below. We measured biliary cholesterol secretion directly in this animal.

Cholesterol secretion by the mass method

Cholesterol secretion was measured in duplicate in four animals by a technique similar to that previously described by Grundy and Metzger (1). Double-lumen tubes were constructed from two size 8 French Pharmaseal 42-in feeding tubes. One tube was 5–7 cm longer than the other. The tubes were sealed within a flexible Silastic® sheath with Silastic® adhesive (Dow-Corning).

The double-lumen tube was passed through a stab wound in the skin and abdominal musculature. The tips were then introduced through an incision into a loop of jejunum and threaded proximally until the more proximal tip was opposite the ampulla of Vater (tubes were in general threaded thus about 10–15 cm). After surgery, the animals wore nylon net monkey jackets attached to flexible metallic tethers. Tubes were led to the outside of the animal's cage through the tethers (jackets and tethers from Alice King Chatham Medical Arts, Los Angeles, CA). Animals recovered from surgery after 4–12 days. They returned to consumption of their maintenance diet and elimination of regular formed stool.

After recovery, animals were fasted overnight prior to measurement of cholesterol secretion. A liquid formula diet was infused through the more proximal tube at a rate sufficient to provide 1/24 of the animals' daily caloric requirement per hour. The liquid formula contained 18% of calories as protein, 38% as safflower oil, and 44% as carbohydrate. The formula contained no cholesterol, but did contain 0.375 g β-sitosterol/liter. It was stirred continuously during each experiment and infused at a constant rate by a Pharmacia peristaltic pump for 8–9 hr. Pumping rates were 0.389 to 0.480 ml/min (approximately 150 μg of β-sitosterol/min) for various studies. Approximately 5 ml of intestinal contents were collected hourly through the more distal tube.

Formula and intestinal contents were analyzed for cholesterol and β-sitosterol mass by gas–liquid chromatography following saponification and hexane extraction (14). Trimethylsilyl ethers of the sterols were prepared and injected onto a glass column packed with DC 560 and installed in a Tracor 560 Gas Chromatograph equipped with a flame ionization detector. 5α-Cholestane was used as an internal standard for quantitation of cholesterol and β-sitosterol (14).

Secretion rate of cholesterol was calculated as follows:

β-Sitosterol input (mg/hr)

$$= \beta\text{-sitosterol in formula (mg/ml)}$$

$$\times \text{ inflow rate (ml/hr).} \quad \text{Eq. 1}$$

Cholesterol secretion (mg/hr)

$$= \beta\text{-sitosterol input} \times \frac{\text{cholesterol (effluent)}}{\beta\text{-sitosterol (effluent)}}. \quad \text{Eq. 2}$$

Studies were routinely performed over a period of 8 hr (except animal #6, study 2, 9 hr). Because of initial gallbladder contraction, the first 3–4 hourly values were generally higher than those found later; therefore, data for the final 4–5 hours of a study were averaged (except animal #6, study 2, last 6 hr).

Cholesterol secretion by the isotope ratio method

For this method about 70 μCi of [1,2-^{3}H]cholesterol was purified by thin-layer chromatography and administered to each animal intravenously 6 weeks before the test. The exact amount of [^{3}H]cholesterol administered was irrelevant as long as it provided measurable isotope in the stool after 6 weeks. The test consisted of feeding a known amount of [^{14}C]cholesterol (0.05 μCi purified by TLC) daily to the animals for 10 days and measuring: a) daily ^{3}H dpm/^{14}C dpm in casual stool collections (fecal isotope ratio); and b) plasma ^{3}H-cholesterol specific activity (three times, at the beginning, middle, and end of the 10-day period).

The daily fecal isotope ratio was determined. For this, 1 gram of stool was saponified with 2 N alcoholic KOH and extracted three times with petroleum ether. The petroleum ether was evaporated and the isotopic steroid was counted in a liquid scintillation counter after separation of neutral steroids from other compounds by TLC (14). For assay of neutral steroids all neutral steroid bands on the thin-layer plate were pooled.

The [^{3}H]cholesterol specific activity was determined in 250 μl of serum extracted with 4.75 ml of 99% isopropanol. The extract was analyzed for total serum cholesterol using Lipid Research Clinics methodology (15);

1.0 ml of the isopropanol extract was also counted. For this, the isopropanol was evaporated, a toluene base scintillation fluid was added, and radioactivity was assayed in a liquid scintillation counter.

In order to determine the biliary cholesterol mass secreted, the following was calculated.

Biliary secretion of [³H]cholesterol (dpm/day)

$$= \text{dietary intake of } [^{14}C]\text{chol. (dpm/day)}$$
$$\times \frac{\text{fecal } [^{3}H]\text{chol. (dpm)}}{\text{fecal } [^{14}C]\text{chol. (dpm)}} . \quad \text{Eq. 3}$$

Biliary secretion of cholesterol (mg/day)

$$= \frac{\text{biliary secretion of } [^{3}H]\text{chol. (dpm/day)}}{\text{plasma } [^{3}H]\text{chol. sp act (dpm/mg)}} . \quad \text{Eq. 4}$$

Equation 3 defines biliary secretion of [³H]cholesterol (dpm/day). Since plasma [³H]cholesterol specific activity decreased in a log-linear fashion throughout the test, biliary secretion of cholesterol mass was estimated using the plasma specific activity found on the previous day (to allow for gastrointestinal transit time). As discussed below (Results), values from the first 5 days were usually discarded since it took this long for the [¹⁴C]cholesterol fed in the diet to equilibrate with the [³H]cholesterol that entered the intestine through the biliary system; therefore, only data from the last 5 days were used for comparisons. Days chosen for analysis are indicated in Fig. 1.

Cholesterol secretion by direct mass measurement

One animal underwent laparotomy and bile duct cannulation. The common bile duct was divided and a Silastic (Dow-Corning) tube was introduced into the proximal and distal ends of the bile duct, creating an extended loop of tubing through which bile flowed freely. This loop of tubing was exteriorized through a stab wound through the skin and abdominal musculature and left intact. At the time of bile duct surgery, a single-lumen tube was also introduced into the small bowel as described above for the mass ratio method. The tip of this tube was positioned opposite the ampulla of Vater. After the animal had recovered from the surgical procedure (10 days), he was lightly anesthetized and the bile duct cannula was divided. A connecting tube was attached to the proximal end. This tube and the intraduodenal tube were both led through a flexible metal tether to the outside of the cage as described above. The distal end of the bile duct catheter was capped for the duration of the secretion study.

For the experiment, Portagen® (Mead-Johnson) was continuously infused through the intestinal tube and bile was collected for 24 hr in hourly intervals. The volume of bile so collected was noted, and an aliquot equal to 5% of the bile flow was reserved. The remainder was returned to the animal through the intestinal tube.

Bile cholesterol was analyzed after extraction with chloroform–methanol 2:1. The dried chloroform extract was redissolved in ethanol, saponified, and extracted a second time with Skelly Solve B. Cholesterol in the Skelly Solve B extract was evaporated, redissolved in isopropanol, and measured by AutoAnalyzer (15). Bile secretion in this animal was measured during a single 24-hr period. The data from the first 5 hr and the last 14 hr of this study were discarded. The first 5 hr were required for equilibration and gallbladder contraction, and observations from this period were twofold higher than those that followed. With time, the removal of 5% of the bile secreted resulted in a small but statistically significant decrease in cholesterol secretion (mean secretion hours 6–10, 2.20 ± 0.17 mg/hr; hours 20–24, 1.53 ± 0.16 mg/hr, $P < 0.01$). We therefore averaged data from only the first 5 hr after equilibration.

Statistical analysis

Secretion rates were associated with body weight by calculation of the correlation coefficient (16). Correlations were also used to compare measurement of cholesterol secretion by the isotope ratio method with that measured by intestinal intubation (mass ratio). Regression coefficients were compared as described in Sokal and Rohlf (17).

RESULTS

Fig. 1 and Table 1 show the hourly secretion of cholesterol measured by the isotope ratio method (IRM). Our previous study of cholesterol absorption in man similarly employed continuous daily feeding of isotopic cholesterol, and in that study it was necessary to discard data from the first 2–3 days of isotope feeding (11). Apparently, equilibration between [¹⁴C]cholesterol entering the intestine from the diet and [³H]cholesterol entering from bile takes 4–5 days. It is not surprising that the equilibration period of the secretion study exceeds that of the absorption experiment, since for the absorption study administration of both isotopes began simultaneously, whereas in the present study isotope exists in bile before administration of isotope in the diet begins. The previous experiment also demonstrated that absorbed isotopic cholesterol was resecreted after 10 days and artificially reduced cholesterol "absorption." For this reason, the present studies of cholesterol secretion were carried out for only 10 days.

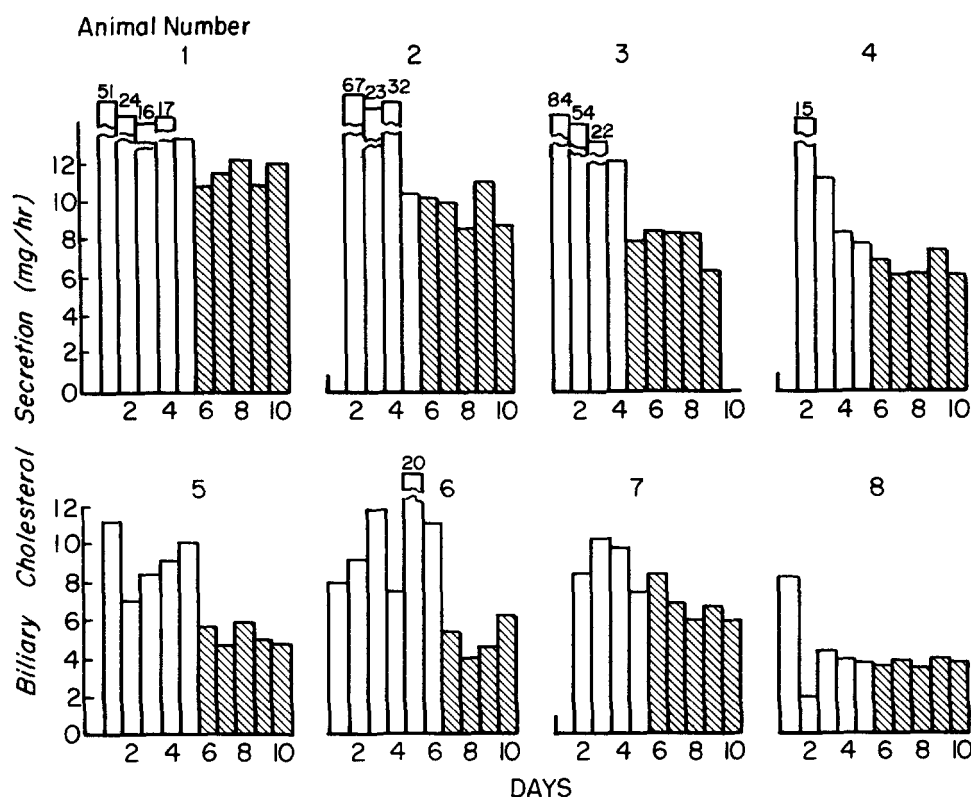Table 1 presents mean values (± standard deviation)

Fig. 1. Biliary cholesterol secretion (mg/hr) for days 1–10 in each of eight animals measured by the isotope ratio method. Values from days indicated by cross-hatched bars were selected to obtain mean ± standard deviation for Table 1.

of cholesterol secretion measured by the isotope ratio method for these eight animals. Data from the first 5 days were routinely discarded. Animal #6 did not consume all the isotope offered for the first five daily doses, and data for the first 6 days on this animal were there-

fore discarded. Biliary cholesterol secretion by IRM varied from 3.6 to 11.4 mg/hr, and the mean coefficient of variation for quintuplicate daily analyses was 9.9%. Table 1 also shows the "direct" mass measurements of cholesterol secretion measured either by in-

TABLE 1. Biliary cholesterol secretion

Animal Number	Weight	Species[a]	Diet[b]	Method Used to Measure Biliary Cholesterol Secretion[c]			
				Isotope Ratio	Mass Ratio 1	Mass Ratio 2	Bile Duct Cannulation
	kg			*mg/hr*			
1	13.1	S.M.	A	11.4 ± 0.6 (5)			
2	11.5	S.M.	A	9.7 ± 1.0 (5)	10.5 ± 2.1 (4)	7.6 ± 1.7 (5)	
3	8.0	S.M.	A	7.8 ± 0.9 (5)	9.5 ± 1.4 (4)	5.1 ± 0.8 (4)	
4	7.9	S.M.	A	6.4 ± 0.6 (5)	5.2 ± 0.8 (4)	7.7 ± 1.7 (4)	
5	6.9	R.M.	A	5.2 ± 0.6 (5)			
6	6.0	R.M.	A	5.0 ± 1.0 (4)	4.3 ± 0.5 (5)	7.8 ± 1.0 (6)	
7	5.7	R.M.	A	6.7 ± 0.9 (5)			
8	5.1	A.G.	B	3.6 ± 0.2 (5)			
9	4.2	A.G.	B				2.2 ± 0.2 (5)

[a] Species: S.M., stumptail macaque (*Macaca speciosa*); R.M., rhesus macaque (*Macaca mulatta*); A.G., African green monkey (*Cercopithecus aethiops*).

[b] Diet, A, fat:carbohydrate:protein calories = 40:40:20; fat source, safflower oil; 0.79 mg cholesterol, 0.06 mg β-sitosterol/kcal. B, fat:carbohydrate:protein calories = 40:41:19; fat source, lard; 0.15 mg cholesterol, 0.18 mg β-sitosterol/kcal.

[c] Method: mean ± SD (n) for cholesterol secretion.

testinal intubation (mass ratio) or by bile duct cannulation.

We compared cholesterol secretion measured by the mass ratio method with that measured by the isotope ratio method in paired experiments in four animals (#2, 3, 4, and 6). These animals all consumed the same diet. For the isotope ratio method we averaged the hourly (= daily/24) secretions on the last 5 days of the isotope study for each animal (last 4 days for animal #6). Comparisons of the duplicate measures of cholesterol secretion by the mass ratio method (Mass 1 vs Mass 2, Table 1) did not reveal a significant difference between the two measures (paired $t = 0.064$, $P > 0.90$), and so we used the mean of these two measures for each animal for the comparison with the IRM. The two methods correlated well in these four animals ($r = 0.97$, $P < 0.05$).

In an attempt to further compare the two methods, we examined the relationship of body weight to secretion measured by both methods (**Fig. 2**). Since the linear regression of secretion measured by the isotope method vs body weight for the four animals used for the paired tests above ($\hat{y} = 0.84X + 0.24$) was the same as that for the remaining four animals (#1, 5, 7, 8; $\hat{y} = 0.85X + 0.15$), we grouped all eight animals together ($\hat{y} = 0.85X + 0.17$; $r = 0.94$, $P < 0.001$). In the four animals used for the paired tests above, we obtained a similar linear regression of cholesterol secretion measured by the mass ratio method vs body weight ($\hat{y} = 0.56X + 2.5$; $r = 0.97$, $P < 0.05$). When we compared the two slopes with one another, the comparison proved nonsignificant ($F_{(1,8)} = 1.42$; $0.25 < P < 0.50$).

We measured cholesterol secretion directly by bile duct cannulation in a ninth animal. This animal weighed less than the other animals (4.2 kg) and secreted less

cholesterol (2.2 mg/hr) (Table 1, Fig. 2). The measured secretion of this 4.2-kg animal was well modelled by the regression of body weight against secretion measured by the isotope ratio method.

DISCUSSION

In 1972, Grundy and Metzger (1) validated a physiological method for estimation of hepatic secretion of biliary lipids in man. Subsequent use of this method enabled investigators to further explore the pathogenesis of cholesterol gallstone disease and the role of various diets, drugs, and genetic factors in biliary lipid secretion (2–9).

The present study evaluates a new method for measuring secretion of one important bile component, cholesterol. It has the advantage of simplicity and affords multiple replicate observations on which statistical evaluation can be performed. Complete fecal collections are not necessary for analysis.

We have presented three lines of evidence to suggest that the new method accurately measures cholesterol secretion. First, the new method correlates well with the accepted method for measuring biliary secretion (1) in paired experiments in four animals. Second, the slope of the regression of cholesterol secretion versus body weight measured by the new method (eight animals) did not differ significantly from that measured by the method of intestinal intubation in the four animals described above. Finally, the isotope method anticipated the relationship of cholesterol secretion to body weight in a ninth animal in which cholesterol secretion was directly measured by bile duct cannulation.

Two problems arise in attempting to draw comparisons between the isotope ratio method and the method of intestinal intubation. First, it is apparent that the coefficient of variation for the daily measures of secretion afforded by the isotope method is small (average coefficient of variation for eight tests in eight animals = 9.9%), whereas the duplicates obtained by repeated analysis using the method of intestinal intubation did not agree as well with one another (mean percent difference for all the studies was 50%). It is unclear why the repeated measures of cholesterol secretion by intestinal intubation did not agree well. There was no consistent trend for the secretion to increase or decrease with time (Mass 1 was consistently measured 10–12 days before Mass 2) or with our experience with the technique. The mean coefficient of variation for the hourly values of the eight individual tests in four animals was 17%, which agrees well with the previous report (1). We anticipated some day-to-day variation (1), and therefore elected to measure bile secretion by the mass ratio
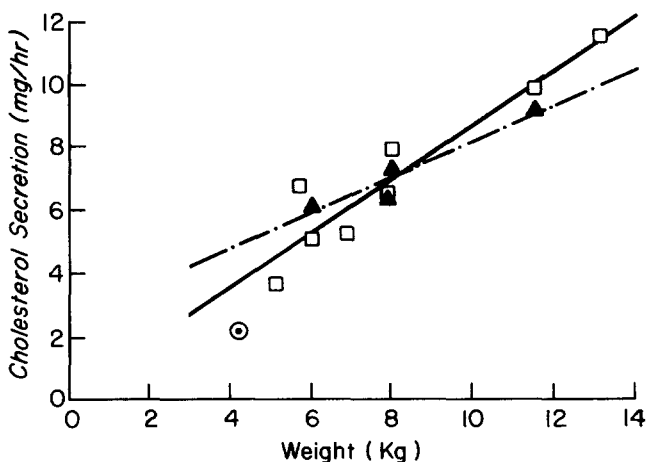

Fig. 2. Cholesterol secretion measured by isotope ratio method (□), intestinal intubation (mean of mass 1 and mass 2 data of Table 1) (▲), or bile duct cannulation (⊙), (— · —), Mass ratio method; (———), isotope ratio method.

method twice, but we found more variation than previously reported. A second concern with the current (IRM) method is that individual daily values probably represent the minimal estimation of the day-to-day variation that really occurs, since unabsorbed cholesterol from several successive daily dietary isotope doses is represented in each stool collection.

Evaluation of the isotope ratio method was undertaken with recognition of four potential pitfalls. *1*) Biliary resecretion of absorbed dietary isotope over the 10 days of the study would artifactually reduce observed cholesterol secretion. Our previous evaluation of an isotope ratio method for measurement of cholesterol absorption in man suggested that dietary cholesterol was not resecreted over the 10 days of the experiment (11), and our experience in the present study was similar. The coefficient of variation for sequential daily analyses of secretion in individual animals ranged from 5.6 to 20% (mean 9.9%). However, an initial 5-day period of equilibration necessitated use of stool samples from only the last 5 days for analysis. *2*) For the present method to accurately measure cholesterol secretion, plasma and biliary cholesterol specific activities must equal one another 6 weeks after an intravenous dose of radiolabeled cholesterol. Henderson and St. Clair (12) have previously shown that these two equilibrate in nonhuman primates consuming diets containing cholesterol such as used in this study. Biliary and plasma cholesterol specific activities following intravenous injection of radiolabeled cholesterol have been shown to closely approximate each other in man (18, 19), although the effects, for example, of a diet free of cholesterol on this equilibration have not been extensively investigated. Administration of bile acid-binding resin eliminates the equality between biliary and plasma cholesterol specific activity in man, however, and would possibly render the present method for measuring cholesterol secretion inaccurate (20). *3*) The present method depends on equal absorption of endogenous (biliary) and exogenous (dietary) cholesterol. Absorption of significantly less exogenous than endogenous cholesterol would lead to underestimation of biliary cholesterol secretion by the IRM. The equality of endogenous and exogenous cholesterol absorption has been assumed in many experimental situations, but direct proof of equality of absorption of these two forms of cholesterol is lacking. The study of Mok, von Bergmann, and Grundy (10) bears on this, since differential absorption of endogenous and exogenous cholesterol in their study would have led to inequalities of cholesterol absorption measured by fecal excretion compared to intraduodenal perfusion. In the present study the isotope ratio method did not consistently underestimate secretion compared to methods that directly assess the mass of cholesterol

secreted. This observation suggests that, at least under the conditions of the present experiment, there were no large differences between absorption of exogenous and endogenous cholesterol. *4*) Finally, exchange of isotopic dietary cholesterol (^{14}C) with unlabeled cholesterol in the intestinal mucosa could artificially cause an overestimation of biliary cholesterol secretion since biliary isotopic cholesterol (^{3}H) and mucosal cholesterol would have similar specific activities. We were concerned with this problem in our previous study that evaluated an isotopic ratio method for measuring cholesterol absorption (11). That method was subject to many of the same potential pitfalls as this one, and our successes with that method encouraged us to undertake the present study. Presumably, the intestinal mucosa becomes labeled to some extent during the extended period of daily administration of isotope in the diet. The period needed to reach that equilibrium no doubt accounts in part for the artifactually high values found during the first 5 days of administration of the isotope. Since no systematic error in the method was found, the use of the values obtained on the last 5 days is apparently valid.▪

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