# Sources of error in the isotopic cholesterol balance method in African green monkeys consuming a cholesterol-free diet

**G. R. Henderson[1] and R. W. St. Clair[2]**

Arteriosclerosis Research Center and the Department of Pathology, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103

**Abstract** Six African green monkeys were labeled intravenously with [1,2-$^3$H]cholesterol while consuming a cholesterol-free liquid formula diet. The plasma cholesterol specific activity was compared with the specific activity of the biliary cholesterol and bile acids and with the fecal neutral steroids in order to determine whether the traditional isotopic balance method was valid for the calculation of endogenous cholesterol excretion. The specific activity of biliary cholesterol and bile acids averaged 10–15% lower than plasma cholesterol specific activity. Fecal cholesterol and coprostanone specific activities were similar to that of the biliary cholesterol, but the specific activity of fecal coprostanol was approximately 25% lower. This suggests that biliary cholesterol and bile acids were derived from a pool of hepatic cholesterol that did not completely equilibrate with the whole body exchangeable cholesterol pool. In addition, there was further reduction in the specific activity of coprostanol, the major fecal neutral steroid, presumably by cholesterol synthesized in the lower intestine and preferentially converted to coprostanol. As a result, the traditional isotopic balance procedure underestimated endogenous neutral steroid excretion by 46% and bile acid excretion by 31% in African green monkeys fed the cholesterol-free diet. Within 7 days after the addition of 1 mg cholesterol/kcal to the diet, the specific activities of plasma and biliary cholesterol and biliary bile acids were identical and there was no difference in the specific activities of the individual fecal neutral steroids. Thus, the traditional isotopic balance procedure (DPM fecal neutral steroids + bile acids/specific activity [DPM/mg] plasma cholesterol) can be used for calculation of endogenous cholesterol excretion in cholesterol-fed animals during the nonsteady state when plasma cholesterol concentrations are rapidly increasing, as well as after a new steady state has been achieved.—**Henderson, G. R., and R. W. St. Clair.** Sources of error in the isotopic cholesterol balance method in African green monkeys consuming a cholesterol-free diet. J. Lipid Res. 1980. **21:** 854–861.

**Supplementary key words** cholesterol absorption · cholesterol excretion · hyporesponsiveness to dietary cholesterol · hyperresponsiveness to dietary cholesterol

Over the past 20 years, various methods have been devised to measure the parameters of whole body cholesterol metabolism (absorption, synthesis, and excretion) in man and experimental animals. These methods are based on chromatographic procedures that require the determination of the mass of fecal steroids (1, 2), isotopic procedures that estimate sterol excretion based on plasma cholesterol specific activity (3), or a combination of the two (4). These procedures all require the complete recovery of fecal steroids (cholesterol and its bacterial degradation products, and bile acids) and, in order to measure cholesterol synthesis, excretion, and absorption, necessitates the subject being in the steady state wherein plasma cholesterol concentrations are constant and whole body cholesterol input equals output.

For several years we have been studying whole body cholesterol metabolism in nonhuman primates that display a wide range of individuality of plasma cholesterol response to dietary cholesterol. Even though a substantial proportion of this individuality in plasma cholesterol response is genetically mediated (5), thus far no major or consistent differences have been found in the parameters of whole body cholesterol metabolism that can adequately explain this individuality in plasma cholesterol response (6–9). Since a number of these studies were done while the animals were in the cholesterol steady state, it is possible that differences in one or more of the parameters of whole body cholesterol metabolism were expressed only during the period when plasma cholesterol concentrations were increasing, after challenge with dietary cholesterol (i.e., the nonsteady state). Once the steady state has been achieved, these differences might no longer be apparent or may not reflect the changes that occurred during the nonsteady state. Although

Abbreviation: GLC, gas–liquid chromatography.

[1] Present address: Division of Toxicology, Food and Drug Administration, Department of Health and Human Services, Washington, DC.

[2] To whom all correspondence should be addressed.



Downloaded from www.jlr.org by guest, on June 19, 2012

some studies have measured cholesterol absorption and excretion by isotopic procedures during the nonsteady state (6–8), none have rigorously validated the isotopic method under the conditions of the nonsteady state in order to assure that the failure to demonstrate significant differences in cholesterol absorption and excretion between hypo- and hyperresponding animals is real, and not the result of methodologic error in the isotopic balance procedure. Thus, the purpose of this study was to determine the validity of the isotopic balance method in African green monkeys (*Cercopithecus aethiops*) during both the steady state, while consuming a cholesterol-free diet, and the nonsteady state in which the plasma cholesterol concentrations were rapidly increasing following the addition of cholesterol to the diet. To do this, we compared the specific activity of biliary and fecal cholesterol and biliary bile acids with plasma cholesterol specific activity in animals labeled with [1,2-$^3$H]cholesterol and consuming first a cholesterol-free diet and then a cholesterol-containing diet.

## MATERIALS

### Animals

Six mature male African green monkeys of the grivet type (*Cercopithecus aethiops*) weighing approximately 5 kg were housed individually in stainless steel cages so that complete fecal collections could be carried out on each animal. These animals were initially prepared surgically by a procedure known as cholecystopexy (10) in which the fundus of the gallbladder was attached to the abdominal wall allowing repeated percutaneous sampling of the bile.

### Diets and plasma cholesterol specific activity

After allowing the animals at least 6 weeks to recover from surgery, they were fed an essentially cholesterol-free liquid formula diet (**Table 1**). This diet provided 40% of calories as fat, 16% as protein, and 42.6% as carbohydrate. In order to assure that this liquid formula diet remained as a stable suspension throughout the day (animals were fed from 8 AM to 4 PM), the addition of 0.6% gelatin was found to be essential. The diet was prepared, mixed in a blender, stored frozen, thawed, and blended again just prior to feeding. The diet was fed ad libitum and measurements of intake were made daily throughout the experiment. This diet maintained the body weight of these animals throughout this experiment and addition of cholesterol to the diet did not affect body weight.

TABLE 1. Composition of liquid formula diet

| Ingredients | g/100 g | kcal/100 g |
|---|---|---|
| Casein | 3.5 | 14.0 |
| Soy hydrolysate | 1.5 | 6.0 |
| Gelatin (unflavored) | 0.6 | 2.4 |
| Methionine | 0.03 | 0.12 |
| Sucrose | 12.0 | 48.0 |
| Dextrin | 3.0 | 12.0 |
| Corn Oil | 4.8 | 43.2 |
| Lard | 1.4 | 12.6 |
| Vitamin mix (devoid of vitamin $D_3$) (plus 1.2 I.U. vitamin $D_3$/100 gm diet) | 0.6 | 2.4 |
| USP XIV Salts Mix | 0.8 | |
| Water | 71.8 | |
| Total | 100.0 | 140.72 |

The control diet contained 0.007 mg cholesterol/kcal derived exclusively from the lard. For preparation of the cholesterol-containing diet, crystalline cholesterol was added to a final concentration of 1.0 mg/kcal by first dissolving the cholesterol in the heated oil.

After 3 weeks of consuming this diet, during which time body weight and plasma cholesterol concentrations remained constant, all animals were administered [1,2-$^3$H]cholesterol intravenously. The [1,2,-$^3$H]-cholesterol (New England Nuclear) was purified by thin-layer chromatography as described previously (9) and was greater than 99% pure after purification. The labeled cholesterol was dissolved in a few microliters of ethanol and added to 2 ml of a stable, intravenous fat emulsion (Upjohn, Kalamazoo, MI) containing 0.3% poloxalkol, 1.2% lecithin, 15% cottonseed oil, and 83.5% water. Each animal was injected with 40 $\mu$Ci of [1,2-$^3$H]cholesterol in 2 ml of this emulsion. Blood was obtained for determination of plasma cholesterol specific activity as described previously (9) at 10 min, 6 hr, 1, 2, 4, and 8 days, and weekly thereafter for a total of 150 days.

Fifty-two days after administration of the labeled cholesterol, 1 mg cholesterol/kcal was added to the diet (Table 1) and the animals continued on this diet for an additional 100 days. During this time, total plasma cholesterol concentrations and plasma cholesterol specific activity were measured weekly.

### Biliary cholesterol and bile acid specific activity

Animals were fasted overnight in order to allow them to sequester the bulk of their bile pool in their gallbladder and 0.5 ml of bile was collected percutaneously through a 22-gauge needle into a 2-ml syringe. The bile was immediately diluted 1:20 with isopropanol and stored at room temperature until analyzed. An aliquot of the diluted bile was saponified in 1 N alcoholic KOH by refluxing the sample for 1 hr and the cholesterol was extracted three times



Downloaded from www.jlr.org by guest, on June 19, 2012

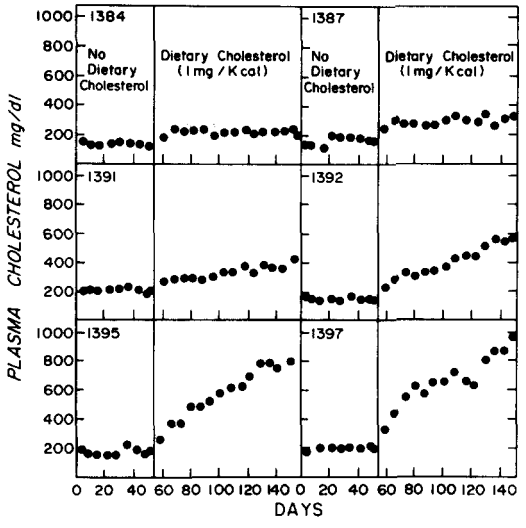


**Fig. 1.** Plasma cholesterol concentrations in the six African green monkeys of this study while consuming the cholesterol-free and the cholesterol-containing liquid formula diets. The animal number is indicated in the upper left corner of each graph.

with redistilled Skellysolve B (Getty Oil Co.). One aliquot was taken for radioactivity determinations and another for cholesterol content (11) in order to allow us to calculate biliary cholesterol specific activity. The remaining aqueous phase containing the bile acids was saponified for an additional 4 hr at 15 psi in 2 N alcoholic KOH, acidified with HCl, and extracted first with twice the volume of choloroform–methanol 2:1 and twice more with chloroform. An aliquot of the pooled chloroform extract was taken for radioactivity determination and another for total bile acid content using the enzymatic steroid dehydrogenase procedure (12), and biliary bile acid specific activity calculated. From these data, we were able to compare biliary cholesterol and bile acid specific activity with that of the plasma cholesterol specific activity.

### Fecal steroid analysis

The methods used for analysis of fecal steroids were identical to those used previously by us (9) and were based on the procedures originally described by Grundy and Ahrens (13). After the individual neutral steroids were separated by thin-layer chromatography, an aliquot was taken for radioactivity and the mass from another aliquot was quantified by gas–liquid chromatography (GLC) on 5 ft × 2 mm internal diameter glass U-tube columns packed with 3% OV-101 on 100/120 Gas Chrom Q. Separations were carried out at 240°C in a Bendix, Model 2500, gas chromatograph using $N_2$ as the carrier gas (40 ml/min.) with an inlet and detector temperature of 290°C. Quantification was carried out using the free fecal steroids with 5α-cholestane as the internal standard. Corrections were made for differences in the relative weight response of the internal standard and the fecal steroids.

All radioactivity determinations were made in a Beckman LS-230 liquid scintillation counter after dissolving the sample in 10 ml of toluene containing 6 g/l of 2,5-diphenyloxazole. Samples were counted to a 2 sigma error of less than 2% and quenching was corrected for by the external standard channels ratio method.

## RESULTS

The mean plasma cholesterol concentration during the period of consumption of the cholesterol-free liquid formula diet was 168 mg/dl and was similar for all animals (**Fig. 1**). Upon addition of 1.0 mg cholesterol/kcal to the diet, there was an increase in the plasma cholesterol concentration of all animals that, depending on the individual animal, ranged from a final concentration of 280 mg/dl to as high as 980 mg/dl (Fig. 1). The most hyperresponsive animals (nos. 1395 and 1397) took as long as 90 days to achieve a relatively stable plateau in plasma cholesterol concentrations, while the plasma cholesterol concentrations of the most hyporesponsive animals stabilized in less than 14 days after addition of cholesterol to the diet.

In order to compare the specific activity of biliary cholesterol and bile acids with that of plasma cholesterol, we measured specific activities at intervals after injection of [1,2-$^3$H]cholesterol. Results are shown in Figs. 2 and 3. Plasma cholesterol specific activity plotted on a semilogarithmic scale versus time described a curve for approximately 21 days and a linear decay thereafter. Upon addition of cholesterol to the diet there was a rapid drop of approximately 20% in plasma cholesterol specific activity that occurred within the first 7 days after initiation of cholesterol feeding (7 days was the earliest time point measured). Thereafter, the plasma cholesterol specific activity decayed in a log-linear fashion.

Biliary cholesterol specific activity (**Fig. 2**) decayed in parallel with that of plasma cholesterol but had an average specific activity 10–15% lower than the plasma cholesterol throughout the period while the animals were consuming the cholesterol-free diet. Within the first 7 days after initiation of dietary cholesterol, the plasma and biliary cholesterol specific activities became equal and remained so throughout the entire period of cholesterol feeding.



Downloaded from www.jlr.org by guest, on June 19, 2012

As shown in **Fig. 3,** the biliary bile acid specific activity increased for the first 15 days after injection of [1,2-$^3$H]cholesterol after which time it decayed in parallel with the plasma cholesterol. During the period while the cholesterol-free diet was being consumed, the bile acid specific activity was consistently from 5–10% less than that of the plasma cholesterol. The specific activities rapidly equalized, however, within 7 days of addition of dietary cholesterol and remained so for the duration of the experiment. Results in Figs. 2 and 3 represent the mean of the six animals whose plasma cholesterol concentrations are shown in Fig. 1. The results from these animals were averaged, since the relationship of the plasma cholesterol, biliary cholesterol, and bile acid specific activities were similar, regardless of whether the animals were hypo- or hyperresponsive to dietary cholesterol.

For the cholesterol-fed animals, the biliary cholesterol and bile acid specific activities were virtually identical with plasma cholesterol specific activities determined on the same day. As a result, it is apparent that the traditional isotopic balance procedure, in which the disintegrations per minute (DPM) of fecal neutral steroids or bile acids are divided by the specific activity (DPM/mg) of the plasma cholesterol to give the mg of endogenous cholesterol or bile acids excreted, would provide a valid measure of endogenous cholesterol excretion. This was true in the cholesterol-fed animals, both during the nonsteady state when plasma cholesterol concentrations were rapidly increasing, as well as after the new steady state had been achieved. Similar results were obtained regardless of whether the animals were hypo- or hyperresponsive to dietary cholesterol.

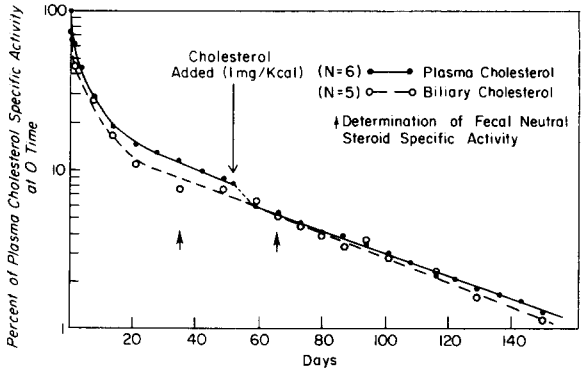


**Fig. 2.** Comparison of plasma and biliary cholesterol specific activity before and after addition of cholesterol (1.0 mg/kcal) to the liquid formula diet. The plasma cholestrol specific activity, 10 min after injection of 40 $\mu$Ci of [1,2-$^3$H]cholestrol is represented as 100%. The plasma cholesterol specific activity curve is the mean of all six animals. The biliary cholesterol specific activity curve is the mean of five animals as we were routinely unable to obtain bile from one animal.

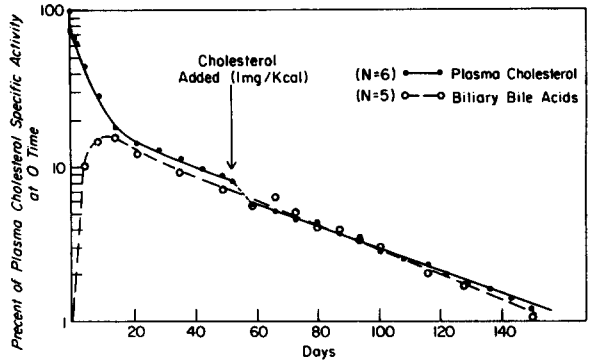


**Fig. 3.** Comparison of plasma and biliary bile acid specific activity before and after addition of cholesterol (1.0 mg/kcal) to the liquid formula diet. Other conditions of this experiment are described in the legend to Fig. 2.

In animals receiving the cholesterol-free diet, however, biliary cholesterol and bile acid specific activities averaged 10–15% lower than plasma cholesterol specific activities. This would result in a significant error in measurement of endogenous cholesterol excretion by the traditional isotopic balance procedure. To evaluate this point further, we compared the specific activity of plasma and biliary cholesterol with that of the fecal neutral steroids in the same animals consuming first the cholesterol-free diet and later the cholesterol-containing diet. The points at which these comparisons were made are indicated in Fig. 2. The actual specific activities of plasma and biliary cholesterol and of the fecal neutral steroids are shown in **Table 2.** The biliary cholesterol specific activity in animals consuming the cholesterol-free diet averaged 36% lower than plasma cholesterol specific activity measured at the same time point. This differential in specific activity was somewhat greater than the 10–15% difference obtained when all points were considered (Fig. 2). Fecal cholesterol and coprostanone specific activities were virtually identical to that of biliary cholesterol, while fecal coprostanol, the major fecal neutral steroid, had a specific activity that averaged 25% lower than biliary cholesterol and 52% lower than plasma cholesterol.

In cholesterol-fed animals, plasma and biliary cholesterol specific activities were virtually identical. Because of the presence of large amounts of unabsorbed dietary cholesterol, the specific activity of the fecal neutral steroids was substantially less than that of the plasma or bile. On the other hand, it is important to note that the three fecal neutral steroids had the same specific activity, unlike that seen for animals consuming the cholesterol-free diet.

In animals consuming no dietary cholesterol, the only source of fecal cholesterol would be of endogenous origin. Thus, by comparing the mass of



Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 2. Specific activity of plasma and biliary cholesterol and fecal neutral steroids before and after addition of cholesterol to the diet

| Animal # | Cholesterol-Free Diet | | | | | Cholesterol-Containing Diet | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Plasma | Bile | Fecal | | | Plasma | Bile | Fecal | | |
| | Cholesterol | Cholesterol | Cholesterol | Coprostanone | Coprostanol | Cholesterol | Cholesterol | Cholesterol | Coprostanone | Coprostanol |
| | *DPM/μmol* | | | | | | | | | |
| 1384 | 3687 | 2552 | 2362 | 2462 | 1834 | 1742 | 1788 | 234 | 210 | 239 |
| 1387 | 2822 | 2259 | 2180 | 1924 | 1439 | 1587 | 1509 | 320 | 189 | 219 |
| 1391 | 2400 | 1667 | 1347 | 1260 | 1145 | 1140 | 1098 | 214 | 153 | 185 |
| 1392 | 3095 | 2048 | 1827 | 2642 | 1543 | 1224 | 1194 | 155 | 258 | 178 |
| 1395 | 4369 | | 2537 | 3740 | 1867 | 1814 | | 224 | | 250 |
| 1397 | 4093 | 2318 | 2302 | 2783 | 1938 | 1644 | 1580 | 177 | 307 | 249 |
| x̄ | 3411 | 2169 | 2093 | 2469 | 1628 | 1525 | 1433 | 221 | 223 | 220 |
| SEM | 312 | 149 | 178 | 342 | 125 | 114 | 127 | 23 | 27 | 13 |

Results are expressed as disintegrations per minute (DPM) per μmol and were obtained 35 days after administration of [1,2-$^3$H]cholesterol while the animals were consuming the cholesterol-free diet, and again in the same animals after they had consumed the same diet containing 1 mg cholesterol/kcal for 12 days. Neutral steroids were measured in the feces collected and pooled over a 3-day period. Plasma and biliary steroid specific activity represent those values measured on Day 2 of the 3-day fecal collection period. On the average, 37% of total fecal neutral steroids were excreted as cholesterol, 57% as coprostanol and 6% as coprostanone.

fecal neutral steroids excreted as measured by GLC with endogenous cholesterol excretion calculated using isotopic procedures, the error produced by the differences in specific activity of biliary and fecal neutral steroids versus plasma cholesterol can be evaluated. Results of such a calculation are shown in **Table 3.** Total mass of fecal neutral steroids, as measured by GLC, averaged 7.31 mg/kg/day. This compares with 3.98 mg/kg/day if calculated by the traditional isotopic procedure using the plasma cholesterol specific activity and 6.17 mg/kg/day if calculated from the bile cholesterol specific activity. Thus, the traditional isotopic procedure underestimated actual neutral sterol excretion by 46% and even underestimated neutral steroid excretion by 16% when corrected for differences in the specific activity of biliary and plasma cholesterol by using biliary cholesterol specific activity.

Since we did not measure the mass of bile acids excreted by GLC, it was not possible to compare excretion as determined by the isotopic procedure with an independently derived method of bile acid quantification. Nevertheless, the difference in specific activity of biliary bile acids and plasma cholesterol resulted

TABLE 3. Mass of fecal neutral steroids and bile acids excreted in African green monkeys consuming a cholesterol-free diet: comparison of total mass excreted as measured by gas–liquid chromatography (GLC) with that calculated using the isotopic procedure from the specific activity of plasma or biliary cholesterol[a]

| Animal # | Total Cholesterol[b] Turnover | Total Neutral Steroid Excreted | | | Total Bile Acids Excreted | |
|---|---|---|---|---|---|---|
| | | GLC[c] | Isotope[d] (Plasma Chol. S.A.) | Isotope[e] (Bile Chol. S.A.) | Isotope[d] (Plasma Chol. S.A.) | Isotope[e] (Bile Chol. S.A.) |
| | | *mg/kg/day* | | | | |
| 1384 | 6.66 | 4.74 | 2.77 | 4.00 | 1.33 | 1.92 |
| 1387 | 12.80 | 9.50 | 5.47 | 6.83 | 2.64 | 3.30 |
| 1391 | 13.15 | 8.36 | 4.59 | 6.61 | 3.33 | 4.79 |
| 1392 | 14.83 | 8.36 | 4.37 | 6.60 | 4.28 | 6.47 |
| 1395 | | 5.64 | 2.79 | | 3.44 | |
| 1397 | 12.63 | 7.28 | 3.85 | 6.80 | 3.03 | 5.35 |
| x̄ | 12.01 | 7.31 | 3.98 | 6.17 | 3.01 | 4.37 |
| ±SEM | 1.39 | 0.74 | 0.44 | 0.54 | 0.40 | 0.80 |

[a] All results are the mean of the analysis of two 3-day fecal collections done 28 and 35 days after administration of [1,2-$^3$H]cholesterol, and while the animals were consuming a cholesterol-free diet.

[b] Total cholesterol turnover represents the sum of neutral steroid excretion (measured by GLC) and bile acid excretion based on bile cholesterol specific activity.

[c] Total neutral steroids excreted as determined by GLC.

[d] Total neutral steroids and bile acids excreted as determined by dividing the DPM fecal neutral steroids or bile acids by the specific activity (S.A.) (DPM/mg) of the plasma cholesterol.

[e] Total neutral steroids and bile acids excreted as determined by dividing the DPM fecal neutral steroids or bile acids by the specific activity (S.A.) (DPM/mg) of the biliary cholesterol.



Downloaded from www.jlr.org by guest, on June 19, 2012

in a 31% lower bile acid excretion (3.01 versus 4.37 mg/kg/day) when calculated, based on plasma cholesterol specific activity compared to that calculated from the specific activity of biliary cholesterol.

## DISCUSSION

Using the traditional isotopic balance procedure, endogenous cholesterol excretion is calculated by dividing the radioactivity (DPM) in the fecal neutral steroids and bile acids by the specific activity of plasma cholesterol (DPM/mg). Thus, for this procedure to be valid the specific activity of the fecal neutral steroids and bile acids must be identical with the specific activity of the plasma cholesterol. Most studies in man in which biliary cholesterol and bile acid specific activity have been compared, have shown this to be true (3, 4, 14–18). In fact, in human beings fed cholesterol-free diets, the specific activity of fecal neutral steroids and bile acids has been shown to be actually somewhat higher than that of plasma cholesterol, due to a several day delay in fecal flow and the recirculation of bile acids in the enterohepatic circulation prior to their excretion in the feces (13). In order to correct for this, the specific activity of plasma cholesterol 2 to 3 days prior to the period of fecal collection is generally used to calculate fecal neutral steroid excretion, and 5 to 7 days prior for fecal bile acid excretion. A similar differential in fecal and plasma cholesterol specific activity did not occur in the African green monkeys of this study. As shown in Figs. 2 and 3, the specific activity of biliary cholesterol and bile acids averaged 10–15% lower than plasma cholesterol specific activity in animals consuming the cholesterol-free diet. This suggests that the biliary cholesterol and bile acids are derived from a pool of newly synthesized cholesterol that has not completely equilibrated with the whole body pool of exchangeable cholesterol. Since the liver is the major site of cholesterol synthesis in the body when no dietary cholesterol is being fed, it is reasonable to suggest that a pool of newly synthesized hepatic cholesterol is preferentially used both for excretion directly into bile and for bile acid synthesis. This is consistent with reports in rats in which a substantial percentage of bile acids (19–21) and biliary cholesterol (21) appears to be derived preferentially from a pool of newly formed cholesterol in hepatic microsomes that does not equilibrate with the whole body exchangeable cholesterol pool. Similar results have been obtained in human beings with a total bile fistula in which 31% of the bile acids and 20% of the biliary cholesterol were derived directly from newly synthesized hepatic cholesterol (22). Consistent with the hypothesis that the dilution of the specific activity of biliary cholesterol and bile acids is the result of liver cholesterol synthesis is the fact that this differential is abolished upon cholesterol feeding where liver cholesterol synthesis is known to be inhibited (23).

If the bile acids were being synthesized in the liver from the same pool of cholesterol as was being secreted into the bile then the specific activity of the biliary cholesterol and bile acids should be the same. The fact that the biliary bile acid specific activity was consistently about 5% greater than biliary cholesterol suggests either that they are not derived from the same percursor pool, or more likely that the difference is the result of the enterohepatic recirculation of bile acids. Thus, bile acids produced from cholesterol of higher specific activity derived several days earlier are retained in the enterohepatic circulation and mix with newly synthesized bile acids to produce a pool of generally higher specific activity than the bile acids being newly synthesized. This phenomenon is known to occur in man (2, 18, 24) in which the specific activity of bile acids frequently exceeds that of plasma or fecal cholesterol.

Another potential source of error is the incomplete mixing of cholesterol and bile acids in the gallbladder with bile newly secreted from the liver. This is highly unlikely, however, for the following reasons. We initially determined by cholecystography, for all of the animals of this study, that the cholecystopexy procedure did not interfere with complete emptying of the gallbladder following a meal. Thus there is no evidence for a residual pool of bile in the gallbladder that does not participate in the enterohepatic circulation. Even if there were a slowly mixing pool of bile in the gallbladder, it would be expected to cause a lowering of bile acid and biliary cholesterol specific activities only initially. As this slowly mixing pool was replaced by bile acids and biliary cholesterol of higher specific activity, it eventually would achieve a specific activity greater than that of the cholesterol and bile acids newly secreted from the liver. There was no evidence that such an increase in gallbladder bile specific activity occurred. In fact, the proportionately lower specific activity of biliary cholesterol and bile acids relative to plasma cholesterol remained constant from about 15 days after injection of the radiolabeled cholesterol until initiation of dietary cholesterol on day 52.

In animals consuming the cholesterol-free diet, it was possible to compare the specific activity of fecal neutral steroids with that of plasma and biliary cholesterol in order to determine whether there was further dilution by cholesterol synthesized in the intestine. The specific activity of fecal cholesterol and



Downloaded from www.jlr.org by guest, on June 19, 2012

coprostanone was similar to that of biliary cholesterol while fecal coprostanol had a substantially lower specific activity. Since coprostanol is a bacterial degradation product of cholesterol (25, 26) it must have arisen from a pool of newly synthesized cholesterol that was secreted directly into the intestine and preferentially converted to coprostanol. The intestine is a known site of active cholesterol synthesis in a number of species. Wilson and Reinke (27) have shown that, in the rat, about one-half of this newly synthesized cholesterol is secreted directly into the intestinal lumen. Much of the newly synthesized cholesterol is not reabsorbed since, in most species, synthesis is most active in the terminal ileum (28–30), whereas cholesterol absorption occurs primarily in the upper small intestine (31). We have no explanation for why the newly synthesized cholesterol from the intestine was preferentially converted to coprostanol, but it could result from differences in the physical form of the newly synthesized cholesterol or differences in the bacteria that populate different areas of the intestine.

An additional factor that could contribute to the lower specific activity of fecal coprostanol might be the ingestion of skin surface sterols, that could either be converted by intestinal bacteria to coprostanol or might themselves cochromatograph with coprostanol. We cannot absolutely eliminate this possibility but, since African green monkeys engage in only a minimum of "fur-licking", particularly when housed in individual cages, it seems unlikely that skin surface sterols could account for the nearly 6 mg/animal/day of sterol that would be necessary to account for this observation.

When cholesterol was added to the diet, the difference in specific activity of fecal coprostanol, cholesterol, and coprostanone disappeared. There are at least three possible explanations for this effect. The first is that dietary cholesterol may inhibit the synthesis of cholesterol by the intestine. This seems unlikely since cholesterol synthesis by the intestine, as reported in rats (28, 32) and squirrel monkeys (33), is not subject to major feedback control by cholesterol. The second possibility is that, as a result of cholesterol feeding, most of the newly synthesized cholesterol is diverted into the lymph rather than the intestinal lumen and is not available to dilute the lumenal cholesterol pool. The third possibility, and the one that seems most likely, is that the same amount of cholesterol enters the lumen from intestinal synthesis as in animals fed a cholesterol-free diet, but that this amount is insignificant compared with the mass of cholesterol in the lumen from dietary sources.

As a result of the dilution of biliary cholesterol and bile acids by newly synthesized hepatic cholesterol, plus the further dilution of fecal neutral steroids by intestinally synthesized cholesterol, the traditional isotopic balance procedure underestimates actual neutral sterol excretion by 46% and bile acid excretion by 31% in African green monkeys fed a cholesterol-free diet. Since the liver is the only site of bile acid synthesis, the isotopic balance procedure can be used to measure bile acid excretion in animals consuming a cholesterol-free diet if biliary cholesterol specific activity rather than plasma cholesterol specific activity is used to calculate bile acid excretion.

In a similar manner, excretion of endogenous cholesterol can be calculated in animals consuming a cholesterol-free diet using biliary cholesterol specific activity rather than the specific activity of the plasma cholesterol. This would not account for the cholesterol synthesized by the intestine. However, since little of this intestinally synthesized cholesterol appears to be reabsorbed and most is converted to coprostanol, it probably should not be considered as part of the rapidly exchangeable cholesterol pool. As a result, the calculation of endogenous cholesterol excretion in animals fed a cholesterol-free diet, using biliary cholesterol specific activity may actually provide a more meaningful estimate of cholesterol excretion from the metabolically important cholesterol pool than would the mass balance procedure.

It should be emphasized that even though the traditional isotopic balance procedure underestimates cholesterol excretion in African green monkeys fed a cholesterol-free diet when calculated using the plasma cholesterol specific activity, it appears to give valid results in cholesterol-fed animals during the nonsteady state when plasma cholesterol concentrations are rapidly increasing in response to cholesterol feeding, as well as after a new steady state has been achieved.

Supported by SCOR grant HL-14164 and by an Institutional National Research Service Award, HL-07115, to G. R. Henderson from the National Heart, Lung, and Blood Institute. We acknowledge the excellent technical assistance of Mrs. Virginia Heaster and the aid of Mrs. Brenda Warner in the preparation of this manuscript.

*Manuscript received 20 November 1979, in revised form 12 March 1980, and in re-revised form 21 April 1980.*

## REFERENCES

1. Miettinen, T. A., E. H. Ahrens, Jr., and S. M. Grundy. 1965. Quantitative isolation and gas–liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.* **6:** 411–424.
2. Grundy, S. M., E. H. Ahrens, Jr., and T. A. Miettinen.



Downloaded from www.jlr.org by guest, on June 19, 2012

1965. Quantitative isolation and gas–liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* **6:** 397–410.
3. Hellman, L., R. S. Rosenfeld, W. Insull, Jr., and E. H. Ahrens, Jr. 1957. Intestinal excretion of cholesterol: a mechanism for regulation of plasma levels. *J. Clin. Invest.* **36:** 898 (abstract).
4. Grundy, S. M., and E. H. Ahrens, Jr. 1966. An evaluation of the relative merits of two methods for measuring the balance of sterols in man: isotopic balance versus chromatographic analysis. *J. Clin. Invest.* **45:** 1503–1515.
5. Clarkson, T. B., H. B. Lofland, Jr., B. C. Bullock, and H. O. Goodman. 1971. Genetic control of plasma cholesterol: studies on squirrel monkeys. *Arch. Pathol.* **92:** 37–45.
6. Lofland, H. B., Jr., T. B. Clarkson, R. W. St. Clair, and N. D. M. Lehner. 1972. Studies on the regulation of plasma cholesterol levels in squirrel monkeys of two genotypes. *J. Lipid Res.* **13:** 39–47.
7. Jones, D. C., H. B. Lofland, T. B. Clarkson, and R. W. St. Clair. 1975. Plasma cholesterol concentrations in squirrel monkeys as influenced by diet and phenotype. *J. Food Sci.* **40:** 2–7.
8. Eggen, D. A. 1976. Cholesterol metabolism in groups of rhesus monkeys with high or low response of serum cholesterol to an atherogenic diet. *J. Lipid Res.* **17:** 663–673.
9. Parks, J. S., N. D. M. Lehner, R. W. St. Clair, and H. B. Lofland. 1977. Whole-body cholesterol metabolism in cholesterol-fed African green monkeys with a variable hypercholesterolemic response. *J. Lab. Clin. Med.* **90:** 1021–1034.
10. Throbjarnarson, B., H. Giler, and K. Morrissey. 1972. A technique for sampling gallbladder bile and some observations on bile lipids in the baboon during pregnancy. *In* Medical Primatology. Proceedings of the 3rd Conference on Experimental Medicine and Surgery in Primates, E. I. Goldsmith and J. Moor-Jankowski, editors. Karger-Basel. 52–57.
11. Rush, R. L., L. Leon, and J. Turrell. 1971. Automated simultaneous cholesterol and triglyceride determination on the AutoAnalyzer II instrument. *In* Advances in Automated Analysis. Technicon International Congress, 1970. E. D. Barton et al., editors. Futura Publishing Co., Mt. Kosco, NY. 503–507.
12. Palmer, R. H. 1969. The enzymatic assay of bile acids and related 3$\alpha$-hydroxysteroids; its application to serum and other biological fluids. *In* Methods in Enzymology. R. B. Clayton, editor. Academic Press, New York. 15: 280–288.
13. Grundy, S. M., and E. H. Ahrens, Jr. 1969. Measurements of cholesterol turnover, synthesis, and absorption in man carried out by isotope kinetic and sterol balance methods. *J. Lipid Res.* **10:** 91–107.
14. Lindstedt, S., and E. H. Ahrens, Jr. 1961. Conversion of cholesterol to bile acids in man. *Proc. Soc. Exptl. Biol. Med.* **108:** 286–288.
15. Avigan, J., and D. Steinberg. 1965. Sterol and bile acid excretion in man and the effects of dietary fat. *J. Clin. Invest.* **44:** 1845–1856.
16. Lindstedt, S. 1962. Equilibration of dietary cholesterol and bile acids in man. *Clin. Chim. Acta.* **7:** 1–4.
17. Wood, P. D. S., R. Shioda, and L.W. Kinsell. 1966. Dietary regulation of cholesterol metabolism. *Lancet.* **2:** 604–607.
18. Quarfordt, S. H., and M. F. Greenfield. 1973. Estimation of cholesterol and bile acid turnover in man by kinetic and analysis. *J. Clin. Invest.* **52:** 1937–1945.
19. Mitropoulos, K. A., N. B. Myant, G. F. Gibbons, S. Balasubramaniam, and B. E. A. Reeves. 1974. Cholesterol precursor pools for the synthesis of cholic and chenodeoxycholic acids in rats. *J. Biol. Chem.* **249:** 6052–6056.
20. Ogura, M., Y. Ayaki, and M. Goto. 1976. Further studies on the possible compartmentation of the precursor pool of cholesterol for biosynthesis of cholic acid in the rat. *J. Biochem.* **80:** 537–545.
21. Long, T. T., III, L. Jakoi, R. Stevens, and S. Quarfordt. 1978. The sources of rat biliary cholesterol and bile acid. *J. Lipid Res.* **19:** 872–878.
22. Schwartz, C. C., M. Bermian, Z. R. Vlahcevic, L. G. Holloran, D. H. Gregory, and L. Sivell. 1978. Multicompartmental analysis of cholesterol metabolism in man: characterization of the hepatic bile acid and biliary cholesterol precursor sites: *J. Clin. Invest.* **61:** 408–423.
23. Gould, R. G., C. B. Taylor, J. S. Hagerman, I. Warner, and D. J. Campbell. 1953. Cholesterol metabolism. I. Effect of dietary cholesterol on the synthesis of cholesterol in dog tissue in vitro. *J. Biol. Chem.* **201:** 519–528.
24. Lindstedt, S. 1959. Cholesterol-bile acid conversion in man. *Federation Proc.* **18:** 274.
25. Snog-Kjaer, A., I. Prange, and H. Dam. 1956. Conversion of cholesterol into coprosterol by bacteria in vitro. *J. Gen. Microbiol.* **14:** 256–260.
26. Danielsson, H., and B. Gustafsson. 1959. On serum-cholesterol levels and neutral fecal sterols in germ-free rats. Bile acids and steroids. *Arch. Biochem.* **83:** 482–485.
27. Wilson, J. D., and R. T. Reinke, 1968. Transfer of locally synthesized cholesterol from intestinal wall to intestinal lymph. *J. Lipid Res.* **9:** 85–92.
28. Dietschy, J. M., and M. D. Siperstein. 1965. Cholesterol synthesis by the gastrointestinal tract: localization and mechanisms of control. *J. Clin. Invest.* **44:** 1311–1327.
29. Dietschy, J. M., and J. D. Wilson. 1968. Cholesterol synthesis in the squirrel monkey: relative rates of synthesis in various tissues and mechanisms of control. *J. Clin. Invest.* **47:** 166–174.
30. Dietschy, J. M., and W. G. Gamel. 1971. Cholesterol synthesis in the intestine of man: regional differences and control mechanisms. *J. Clin. Invest.* **50:** 872–880.
31. Swell, L., E. C. Trout, Jr., J. R. Hopper, H. Field, Jr., and C. R. Treadwell. 1958. Mechanism of cholesterol absorption. I. Endogenous dilution and esterification of fed cholesterol-4-$C^{14}$. *J. Biol. Chem.* **232:** 1–8.
32. Siperstein, M. D., and M. J. Guest. 1960. Studies on the site of the feedback control of cholesterol synthesis. *J. Clin. Invest.* **39:** 642–652.
33. Wilson, J. D. 1968. Biosynthetic origin of serum cholesterol in the squirrel monkey: evidence for a contribution by the intestinal wall. *J. Clin. Invest.* **47:** 175–187.



Downloaded from www.jlr.org by guest, on June 19, 2012