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Validation of the Dual-Isotope Plasma Ratio Technique as a measure of Cholesterol Absorption in Old and New World Monkeys[1] (38645)

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The serum cholesterol concentration is the result of ill-defined regulatory processes involving tissue fluxes, lipoprotein transport, synthesis and absorption, and excretion of the sterol from the body. Cholesterol absorption is particularly significant since it is a major determinant of cholesterol synthesis (1, 2) and since bile acids, a major pathway of cholesterol excretion, themselves regulate cholesterol absorption (3) and perhaps synthesis (1). Furthermore, recent indications are that cholesterol absorption may not be as limited in man (4, 5) as originally suggested (6) and thus may contribute significantly to diet-induced hypercholesterolemia.

Unfortunately, methods for determining cholesterol absorption have been traditionally difficult, necessitating complete fecal collections and analysis. Furthermore, no balance method has adequately dealt with the problem of distinguishing unexcreted cholesterol from that actually entering the circulation. It is the latter which has been given pathologic significance as the potentiator of atherosclerosis.

Zilversmit has devised a simple method for cholesterol absorption in the rat using a single plasma sample following simultaneous isotopic doses of orally and intravenously administered cholesterol (7). Thus, cumbersome fecal collection is avoided and an actual measure of cholesterol contributing to the circulating pool by absorption is obtained. The present study was an attempt to validate the dual-isotope method in New and Old World monkeys. Distinct differences have been described in the susceptibilities of these primate species to diet-induced hyperlipidemia (8), and it is possible that cholesterol absorption and reabsorption of endogenous cholesterol may have a bearing on these species differences.

Materials and Methods. Isotopes. Cholesterol-4-^{14}C (52 mCi/mmole), cholesterol-1,2-^{3}H (53 Ci/mmole) and β-sitosterol-22,23-^{3}H (32 Ci/mmole) were obtained from New England Nuclear and purified prior to use by thin layer chromatography on silica gel H developed in the solvent system hexane: diethyl ether (50:50).

Preparation of isotope doses. ^{3}H or ^{14}C-cholesterol for injection (20–40 μCi) was dissolved in 50 μl of 95% ethanol and dispersed in 0.5 ml 0.9% sterile saline. The resulting suspension was injected immediately into the femoral vein of squirrel and cebus monkeys or the cephalic vein of rhesus monkeys. The oral dose of ^{3}H or ^{14}C-cholesterol (30–50 μCi) was similarly dissolved and dispersed in 2.0 ml of 0.9% sterile saline. This is in contrast to the method as initially described by Zilversmit (7), for it was desirable in our strict dietary studies to avoid both a specific fat as carrier and significant amount of exogenous cholesterol. When a carrier dose of cholesterol was included for study of one monkey, the isotopic cholesterol and 30 mg of crystalline cholesterol were dissolved in 700 mg of triolein (Sigma Chemical Company) and this mixture was sonicated in 3.2 ml of 6.8% skim milk powder prior to gavage (7). For simultaneous oral administration of ^{14}C-cholesterol and ^{3}H-β-sitosterol, 5 μCi of each sterol was prepared and intubated as described above. After isotope administration, syringes and tubing were assayed for residual activity and the actual dose administered was appropriately adjusted.

Monkeys. All the animals used in this

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study were born and raised in our primate nursery (9). The eight squirrel monkeys (*Saimiri sciureus*), six cebus monkeys (*Cebus albifrons* and *apella*) and five rhesus monkeys (*Macaca mulatta*) were of both sexes and had been fed their respective formula diets essentially from birth, initially in liquid then in solid form, for periods of 18–30 mo with the exception of one rhesus monkey (No. 14) which was only 6-mo old at the time of study. These diets contained safflower and coconut oil as previously described (1) or were the same diet with corn oil as the dietary fat. Since the diets contained no cholesterol, the calculated absorption values represent reabsorption of endogenous cholesterol.

The monkeys were fasted for 18 hr prior to dosing and for 2 hr thereafter. Prior to a cut-down for intravenous injection, the animals were anesthesized with Halothane (Ayerst Laboratories). The oral dose was administered by stomach tube as they recovered from the anesthesia. Fecal collection pans were placed in the cages immediately thereafter and feces were collected for 5–7 days.

Isotope measurement. Fasting blood samples for determination of specific activity were taken from the femoral vein at 1, 2, 4 and 7 days and at weekly intervals thereafter until the precision of isotope measurement declined significantly. Plasma samples were saponified in alcoholic KOH overnight at room temperature and sterol extracted with hexane prior to counting. Cholesterol was determined by the method of Carpenter *et al.* (10). Fecal samples were homogenized and neutral sterols extracted according to Miettinen *et al.* (11).

^{3}H and ^{14}C in plasma and fecal extracts were determined by liquid scintillation counting in toluene (POPOP). Correction for quenching was applied by the channels ratio method.

Calculations. The ratio of the orally administered to the intravenously administered isotopes in plasma samples was used as a measure of cholesterol absorption after correction for unity of dose (7).

Results and Discussion. A validation of the plasma isotope ratio as a measure of cholesterol absorption necessitates fecal analysis to recover the cholesterol calculated as unabsorbed. Table I compares the plasma isotope ratios of six squirrel, four cebus, and three rhesus monkeys with the fecal recoveries of orally administered isotope. Six additional monkeys have been included as an evaluation of the isotope ratio alone.

Some delay in stabilization of the plasma ratios was apparent in all cebus and rhesus monkeys and in some squirrel monkeys (Table I). The initial decrease in the ratio suggests a delayed hepatic release of the intravenous dose of colloidal cholesterol. It has been previously shown that this form of injected cholesterol is metabolized as lipoprotein cholesterol after initial phagocytosis and assimilation by the liver (12). After this initial stabilization of 2–4 days, constant ratios were maintained indicating that a sample taken 5 days after dosing should be representative.

Concern that delay in release of the intravenous dose could invalidate the absorption ratios prompted the measure of the ratio of the areas under the specific activity time-curves describing the disappearance of the two isotopes since this should take into account delays in release. Table II shows that absorption ratios were similar whether calculated by this graphic procedure or by direct calculation of the ratio of the two labels in plasma.

In Table I, recovery of orally administered cholesterol as estimated by summing the % derived from absorption ratios and the % neutral sterol recovered in the feces indicated that if isotope ratios represented actual absorption, then loss of the oral dose was considerable. The low recoveries in squirrel monkeys three to six may be partially artifactual as extraction methods had not been standardized at that stage. Subsequently, recovery of added ^{14}C-cholesterol to unlabeled fecal homogenates was complete, indicating that the problem did not involve the extraction procedure. Similarly, no major mechanical losses during the 5- to 7-day fecal collection appeared to have occurred and daily analysis of neutral sterol radioactivity indicated that 99% of

TABLE I. COMPARISON OF THE % CHOLESTEROL ABSORBED BY PLASMA ISOTOPE RATIO AND FECAL METHODS IN MONKEYS.

Monkey no.	Species	Diet[a]	Plasma ratio[b] 1	2	4	7	Unabsorbed fecal cholesterol[c]	Recovery[d]
3	Squirrel	Coconut oil	25.4	29.7	28.3	29.6	7.8	37.4
4	Squirrel	Coconut oil	7.9	9.8	10.4	8.7	9.4	18.1
5	Squirrel	Safflower oil	31.3	34.0	32.1	34.7	8.6	42.8
6	Squirrel	Safflower oil	28.1	27.1	26.5	30.6	11.3	41.9
16	Squirrel	Coconut oil	36.3	39.5	36.9	35.8	18.4	54.2
17	Squirrel	Safflower oil	117.0	62.1	40.9	42.3		
18	Squirrel	Coconut oil	231.1	116.0	70.0	73.0		
30	Squirrel	Coconut oil	45.7	42.2	39.4	45.1	23.1	68.1
7	Cebus	Safflower oil	54.2	50.0	47.0	48.2	15.9	64.1
8	Cebus	Safflower oil	64.6	58.6	51.2	49.1	16.4	65.6
9	Cebus	Coconut oil	59.4	50.2	48.0	45.8	8.9	54.7
10	Cebus	Coconut oil	72.8	64.6	59.8	55.0	5.5	60.5
15	Cebus	Coconut oil	80.6	67.5	60.0	59.2		
26	Cebus	Safflower oil	105.6	97.5	72.2	69.4		
12	Rhesus	Corn oil	64.5	61.2	56.4	57.0	9.2	66.2
13	Rhesus	Corn oil	55.2	50.0	44.7	48.9	14.1	63.0
14	Rhesus	Coconut oil	115.0	69.2	69.3	72.6	9.3	81.9
23	Rhesus	Corn oil	54.5	43.5	35.8	33.8		
24	Rhesus	Coconut oil	97.6	76.8	59.3	57.9		

[a] Corey *et al.* (8).

[b] Percentage ratio of orally administered: Intravenously administered isotopic cholesterol in plasma 1, 2, 4 or more than 7 days after dosing.

[c] Percentage oral dose recovered in neutral sterol in 5-day fecal pool.

[d] Percentage total oral dose accounted for by summing the % absorbed at day 7 with the % recovered as neutral sterol in 5-day fecal pool.

all label recovered was found within the first 2 days in all species. Thus, bulk intestinal delay was probably not a factor.

Several other potential explanations for the poor recovery were examined: (a) the possibility of tritium exchange, since the oral dose was usually ^{3}H-cholesterol, and (b) the possibility of exchange of labeled cholesterol in the intestinal tract with unlabeled cholesterol due to the absence of carrier cholesterol. In monkey No. 16, the oral dose was switched to ^{14}C-cholesterol, yet recovery was still only 54% thus minimizing the possibility of isotopic hydrogen exchange. In monkey No. 30, the oral dose of ^{3}H-cholesterol was administered as a skim milk emulsion along with 30 mg crystalline cholesterol. Recovery was only 68%, making it unlikely that cholesterol exchange accounted for the fecal loss.

Another source of error might have been bacterial degradation of the steroid nucleus (13, 14). This was also examined using

TABLE II. COMPARISON OF CHOLESTEROL ABSORPTION CALCULATED BY THE RATIO OF AREAS UNDER DISAPPEARANCE CURVES AND BY SINGLE SAMPLE PLASMA RATIO.

Monkey no.	$\frac{\text{Oral}}{\text{Intravenous}} \times 100$[a]	$\frac{\text{Oral area}}{\text{Intravenous area}} \times 100$
3	29.6	22.0
4	8.7	8.5
5	34.2	36.7
6	30.6	33.0
17	42.3	39.4
18	73.0	86.0
7	48.2	45.0
12	57.0	56.2
14	72.6	66.5

[a] Ratio calculated as the mean of plasma ratios after 4 days.

β-sitosterol, a plant sterol which is poorly absorbed in man and monkeys (4, 15, 16) but which has been shown to be subject to the same degradative losses as the cholesterol

molecule (4, 13, 15, 17). Simultaneous oral administration of radiolabeled sitosterol and cholesterol has been shown to be a valid method for determining cholesterol absorption, automatically correcting for degradation (4, 5).

In the present study, two squirrel and two cebus monkeys were tested 4 mo apart by both absorption methods. Neither cholesterol nor sitosterol carriers were included in the gavage. Only 50–60% recovery of oral sitosterol was obtained, but when isotopic cholesterol excretion was corrected by this sitosterol-loss factor, the calculated cholesterol absorption agreed well with the previously determined plasma ratios (Table III). It thus seemed probable that despite unexplained fecal losses, the ratio of simultaneously administered oral:intravenous doses of radioactive cholesterol in plasma taken at least 5 days after dosage was providing a precise measure of the cholesterol absorption in these monkeys.

The loss of the oral dose is unexplained, but may be related to the fact that cholesterol-free diets were fed. Thus, a tracer dose of isotopic cholesterol (as well as sitosterol) could readily be "lost" via exchange with endogenous cholesterol. A continuous dietary load of cholesterol may be required to saturate endogenous intestinal cholesterol pools and protect against this exchange.

Zilversmit and Hughes (18) designed experiments to validate the plasma isotope ratio method for cholesterol absorption in rats. Using a semi-synthetic or chow diet containing negligible amounts of cholesterol, they obtained a range in cholesterol absorption from 33 to 81%, about the range encountered in these monkeys. However, in the rat studies recovery of isotope was usually greater than 90%. The poor recoveries in the primate experiments require further investigation, since it does not apparently occur in rats.

A comparison between endogenous cholesterol absorption, as determined here with cholesterol-free diets, and absorption of added dietary cholesterol has not yet been studied in these monkeys. Since the amount of cholesterol absorbed is a function of the dietary cholesterol (5), it is likely that the percent of cholesterol absorbed will vary, but species comparisons should remain valid.

The degree of absorption of endogenous and exogenous cholesterol as well as the comparability of various published methods is specifically examined in Table IV. Cholesterol absorption in three species of monkeys is compared in cholesterol-fed animals studied independently via isotopic balance techniques (15, 19, 20) with the monkeys described in the present study. It should be mentioned that the low absorption recorded in rhesus monkeys by Manning *et al.* (20)

TABLE III. COMPARISON OF CHOLESTEROL ABSORPTION BY PLASMA ISOTOPE RATIO AND BY β-SITOSTEROL CORRECTION METHODS.

Monkey no.	Species	Plasma ratio method (%)	Fecal recovery β-sitosterol method[a] (%)
3	Squirrel	29.6	31.8
16	Squirrel	35.8	26.3
7	Cebus	48.2	40.0
10	Cebus	55.0	61.4

[a] 1 − [fecal isotopic cholesterol/fecal isotopic β-sitosterol] × 100. Isotopes administered as a single oral dose.

TABLE IV. COMPARISON OF ENDOGENOUS AND EXOGENOUS CHOLESTEROL ABSORPTION IN MONKEYS DETERMINED BY SEVERAL METHODS.

Species	Diet	% Absorption	Method	Reference
Rhesus	Cholesterol-free	55	Plasma isotope ratio	Corey and Hayes
Rhesus	0.35% cholesterol	48	Isotopic balance	Eggen (19)
Rhesus	0.50% cholesterol	29	Isotopic steady state	Manning *et al.* (20)
Cebus	Cholesterol-free	54	Plasma isotope ratio	Corey and Hayes
Squirrel	Cholesterol-free	36	Plasma isotope ratio	Corey and Hayes
Squirrel	0.35% cholesterol	42	Isotopic balance	Eggen (19)
Squirrel	0.22% cholesterol	55	Isotopic balance	Lofland *et al.* (15)

may be partially a function of methodology (5). Nonetheless, the results are variable and without definite relationship to diet or technique, indicating that the plasma dual-isotope method may provide as valid a measure of cholesterol absorption as more difficult and laborious balance techniques.

Summary. Due to the experimental importance of being able to assess cholesterol absorption during the study of atherogenesis in primates, the plasma isotope ratio technique developed for rats by Zilversmit (7) was tested in New and Old World monkeys and compared with a more cumbersome procedure involving fecal collection and analysis. Although fecal analysis demonstrated an unaccountable loss of the intubated isotope, the comparability of the plasma ratios to absorption calculated by other methods suggests that this technique is probably as reliable as many presently available. The loss of isotope may reflect the absence of dietary cholesterol.

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