Validation of a dual-isotope plasma ratio method for measurement of cholesterol absorption in rats

D. B. Zilversmit[1] and L. Barry Hughes

Graduate School of Nutrition and Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14850

Abstract Several methods for measuring cholesterol absorption in the rat have been compared. After administration of an oral dose of labeled cholesterol (^{14}C or ^{3}H) and an intravenous dose of colloidal labeled cholesterol (^{3}H or ^{14}C) the ratio of the two labels in plasma or whole blood 48 hr or more after dosing compared closely to the ratio of areas under the respective specific activity–time curves. The area ratio method is independent of a time lag between the appearance of oral and intravenous label in the bloodstream. Both measures of cholesterol absorption agree fairly well with a method based on measuring the unabsorbed dietary cholesterol in a pooled fecal sample. The plasma isotope ratio method gave more reproducible results than the fecal collection method when the measurement was repeated in the same animals 5 days after the first measurement. Cholesterol absorption was overestimated by the use of Tween 20-solubilized labeled cholesterol for the intravenous dose. The plasma disappearance curves of injected labeled colloidal cholesterol and cholesterol-labeled chylomicrons infused intravenously over a 3.5-hr period in the same animal coincided within experimental error from the first day until 75 days after injection. The plasma isotope ratio method for cholesterol absorption gave the same results in rats practicing coprophagy as in those in which this practice was prevented. The addition of sulfaguanidine to the diet lowered cholesterol absorption as measured by the plasma isotope ratio to the same degree as that measured by the fecal collection method.

Supplementary key words fecal labeled sterols · colloidal cholesterol · Tween 20-solubilized cholesterol · production rates · sulfaguanidine · chylomicrons

In a previous study (1) we developed a technique for calculating cholesterol absorption in the intact rat from the plasma isotope ratio of two labeled cholesterol doses, one given orally and one intravenously. This procedure is simple, independent of fecal sterol excretion measurements, and agrees with absorption calculated from fecal excretion data. Experiments reported here further validate the plasma isotope ratio method by comparing it to two other methods under a variety of experimental conditions. By comparing these methods, we have examined the following questions. Can the plasma isotope ratio method be applied more than once in the same animal with good precision? Is the plasma isotope ratio affected by coprophagy or its prevention? Is the validity of the plasma isotope ratio method dependent on the form in which the intravenous dose is administered?

In other studies (2–4) it has been shown that labeled, intravenously injected, colloidal cholesterol is quickly removed from plasma, mostly by the Kupffer cells of the liver, and then gradually released into the bloodstream so that over a 4-day period the specific activity–time curve corresponded in shape to that generated by an oral dose of cholesterol. In the following studies we have compared the disappearance from plasma of colloidal cholesterol to that of cholesterol solubilized by Tween and to chylomicron cholesterol.

METHODS

Isotopes

[4-^{14}C]Cholesterol and [1α,2α-^{3}H]cholesterol were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Each isotope was purified by thin-layer chromatography on silica gel H with the solvent system hexane–diethyl ether 1:1 (v/v). The purified materials were then tested by thin-layer chromatography on silica gel G, $AgNO_3$ 2% (w/w), with chloroform as a solvent (5), and by reverse-phase chromatography on kieselguhr G (6). In both systems more than 98% of the radioactivity migrated with a digitonin-precipitated, dibromide-purified cholesterol preparation that was used for carrier.

Preparation of oral and intravenous doses

[^{3}H]- or [^{14}C]cholesterol (0.14–4 μCi) containing

[1] Career Investigator of the American Heart Association.

10–35 μg of cholesterol was dissolved in 0.025 ml of 95% ethanol. Colloidal cholesterol was prepared by adding either 0.5 or 1 ml of 0.9% NaCl. The resulting mixture was injected intravenously within 2 hr of preparation. In one experiment the cholesterol was solubilized in Tween 20 according to Whereat and Staple (7).

The oral doses of [^{3}H]- or [^{14}C]cholesterol (0.6–14 μCi) were prepared by dissolving 20 mg of cholesterol in 520 mg of triolein (grade II, Sigma Chemical Co., St. Louis, Mo.). The oil phase was then suspended by sonication in 8 ml of water containing 25 mg of bile salt (67% sodium taurocholate, Difco Laboratories, Detroit, Mich.). The volume was adjusted with water such that each animal, when given 1 ml by stomach tube, received 2 mg of cholesterol/100 g of body weight. In a few animals (see Table 3) the oral dose contained 4 mg of cholesterol/100 g of body weight.

Chylomicrons labeled with [^{3}H]- or [^{14}C]cholesterol were obtained from thoracic duct lymph of rats fed 6.8 mg of cholesterol dissolved in 177 mg of triolein suspended in 0.8 ml of skim milk (1). The lymph was defibrinated and injected into recipient animals by constant infusion into the jugular vein. Infusion at 1.13 ml/hr was completed in 3.5 hr. The total amount of chylomicron triglyceride infused varied between 28.8 and 57.8 mg in rats weighing between 350 and 500 g. The percentage of labeled cholesterol present in the chylomicron fraction of whole lymph ($S_f > 400$) was determined by layering 4 ml of whole lymph, adjusted to d = 1.063, under a gradient made of 2 ml d = 1.041, 2 ml d = 1.019, 2 ml d = 1.006, and 2 ml of water. In three different lymph preparations used in this study 86.1, 90.0, and 90.7% of the labeled cholesterol was found in the top layer after 45 min of centrifugation in a SW-41 (Beckman) rotor at 40,000 rpm.

Animals

Male Sprague-Dawley rats weighing 150–500 g were obtained from Holtzman Co. or ARS/Sprague-Dawley (Madison, Wis.). In most experiments animals were fed a semisynthetic diet of 26% casein, 55% Cerelose, and 10% triolein, plus vitamins and minerals (8), for a period of 3–10 days before the experiment. They were maintained on the same diet throughout the experiment. The cholesterol concentration in this diet was 0.003%, and animals eating approximately 15 g/day would consume 0.5 mg of cholesterol. In one experiment (Table 4) animals were fed either rat and mouse diet 5RF (Agway, Inc., Syracuse, N.Y.) or a diet composed of 1500 g of the semisynthetic diet suspended in 650 ml of water in which 20 g of agar and 1 g of sorbitol had been dissolved (9).

All animals were fasted for 18 hr prior to oral and intravenous dosing with the isotopes. Fasting was continued for an additional 6 hr unless otherwise noted. The oral isotope dose was given by stomach tube followed immediately by the intravenous isotope dose, which was injected into the external jugular vein of rats under ether anesthesia. Blood samples (approx. 0.3 ml) were obtained from the tail vein and collected in heparinized cups.

Feces were collected in plastic tail cups (10) or in pans below the screened cage bottoms. Feces were removed each day and kept frozen until they were analyzed as 4-day pooled samples.

Calculations

In most experiments the percentage of an oral dose of cholesterol absorbed was measured by two independent methods: from the plasma isotope ratio and from fecal excretion of neutral sterols. The plasma isotope ratio is defined as:

$$\frac{\text{percentage of the oral dose in an aliquot of plasma}}{\text{percentage of the intravenous dose in the same aliquot of plasma}} \times 100$$

This ratio was usually measured at 48, 72, or 96 hr after isotope administration or as the average of such ratios if more than one blood sample was taken. Cholesterol absorption was also calculated from the relationship: 100 − percentage of the orally administered isotope found in the neutral sterols of a 4-day pooled fecal sample. Theoretically, this fraction should be corrected for any neutral sterol degraded in the lower portion of the gastrointestinal tract and for excretion of isotopically labeled neutral sterol derived from the absorbed oral dose. In a previous study (1) these corrections were found to be of the order of 5% and to act in opposite directions, so that for the present studies no corrections for these two factors were made.

In one experiment (Table 4), cholesterol absorption after dual isotope administration was measured by an area ratio method. Instead of using the isotope ratio of oral to intravenously administered cholesterol at a single time point, cholesterol absorption may be calculated from the ratio of areas under the plasma cholesterol specific activity–time curves generated by the oral and intravenous doses from the time of dosing to infinity. This method is based on the following considerations. Let K = fraction of oral dose absorbed, R = rate of irreversible loss of steroid from the body, and S = specific activity of plasma cholesterol (S_o for the orally administered isotope and S_i for the intravenously administered isotope). If S is a valid measure of the specific activity of all steroid secreted irreversibly into the intestinal lumen, then the amount of tracer lost from the body during a small interval, Δt, is equal to $R \times S \times \Delta t$. Between time zero and infinity, the total amount of isotope that entered the body from the intravenous dose will be lost ($R \int_0^\infty S_i dt =$ intravenous dose). However, only that fraction of the oral dose which was

absorbed will be similarly removed ($R \int_0^\infty S_o\, dt$ = oral dose $\times K$). Thus,

$$K = \frac{\int_0^\infty S_o dt}{\int_0^\infty S_i dt} \times \frac{\text{intravenous dose}}{\text{oral dose}}$$

or, for equal intravenous and oral isotope doses:

$$K = \frac{\text{area under the specific activity curve for the oral dose}}{\text{area under the specific activity curve for the intravenous dose}}$$

Because the specific activities are integrated, this calculation is valid even when there is a delay between the entries of the intravenous and oral isotope doses into the tissue spaces.

In the long term turnover studies with labeled colloidal cholesterol and with cholesterol-labeled chylomicrons, the fecal excretion of endogenously labeled steroid was calculated from the amount of labeled steroid derived from the intravenous dose found in feces on 4 separate days during the 11th wk after isotope administration. The radioactivity in each daily fecal steroid fraction was divided by the specific activity of plasma cholesterol on that day. No correction was made for the change in plasma cholesterol specific activity from the time of excretion of labeled cholesterol or bile acid into the intestinal lumen until the time of fecal collection, since separate experiments had shown such a correction to be of the order of only a few percent. It should be noted that the value for steroid excretion calculated in this manner does not include excreted sterols derived from intestinal mucosa before these sterols were equilibrated with the plasma cholesterol (11). For our purposes this omission is an advantage because we wished to compare the cholesterol excretion with production rates calculated from cholesterol disappearance curves. These rates also do not include the fraction of intestinal sterol that is lost before equilibrating with plasma cholesterol.

Production rates of cholesterol were calculated by input–output analysis (12), with one modification: the area under the plasma cholesterol specific activity curve was measured in two steps. The area during the first day after isotope injection was obtained from one-half the product of the 24-hr specific activity and 1 day. This represents a triangle with a base of 1 day and a hypotenuse connecting the 24-hr specific activity point with zero. This modification allows for the observation that colloidal cholesterol is cleared from the circulation in a few minutes and that label reappears[2] over a period of 12 to 24 hr (2). The area beyond the 24-hr point was measured by integration between 1 day and infinity of a three-exponential fit obtained from a nonlinear least square routine on a computer.

Chemical and isotopic analyses

Whole blood or plasma samples were saponified according to Abell et al. (13), and sterol was extracted into hexane. Cholesterol was determined by the method of Zak et al. (14). An aliquot of the hexane extract was evaporated under nitrogen, and ^3H or ^{14}C was determined by liquid scintillation counting in a phosphor solution containing 0.4% PPO and 0.01% POPOP in toluene.

Feces were homogenized in chloroform–methanol 1:1 (v/v) or in water and then extracted with the solvent mixture. The extracted lipid was saponified by the procedure of Borgström (15), and neutral sterols were extracted with one or two portions of hexane so that extraction was 98% complete or better.

Total fecal radioactive steroid in the long-term turnover studies was determined by combustion of fecal samples in a biological oxidizer (R. J. Harvey Instrument Corp., Hillsdale, N.J.) and counting of ^3H_2O in a dioxane-based solution (16) and $^{14}CO_2$ in a modified phenethylamine-based solution (17) containing 250 ml of scintillation grade phenethylamine (Eastman Kodak, Rochester, N.Y.), 350 ml of ethylene glycol monomethyl ether, 400 ml of toluene, 6 g of PPO, and 0.2 g of POPOP.

RESULTS

Plasma isotope ratios

In a previous publication on the dual isotope method for the measurement of cholesterol absorption, the oral dose of cholesterol was administered as an oil emulsion in skim milk (1), and several of the animals had to be eliminated from the study because of diarrhea. In subsequent experiments bile salts were used as a stabilizer for the emulsion. **Table 1** shows cholesterol absorption in two rats with the original milk formula and in six rats with the cholesterol–triolein–bile salt emulsion. The plasma isotope ratios are similar to those obtained previously (49 ± 9) and appear to be approximately the same for the animals given the two different emulsions.

In three of the animals the isotope ratios of plasma and of whole blood were compared at 24, 48, and 96 hr after dosing. Apparently, the cholesterol of the intravenous and oral doses distributes between red blood cells and plasma in a similar manner, because the values for cholesterol absorption calculated from the plasma and whole blood isotope ratios in Table 1 agree closely.

[2] The use of the 24-hr specific activity for the apex of the triangle assumes that the true maximum specific activity occurs at that point. Although for colloidal cholesterol this assumption introduces little or no error, data describing the plasma cholesterol specific activity during the first 24 hr after injecting cholesterol-labeled chylomicrons are not available. However, even if the area during the first 24 hr were underestimated by a factor of 2, the error in the area between time zero and infinity due to this source would be only 5%.

TABLE 1. Comparison of plasma isotope ratios[a] at different times after isotope dosage

Rat	Oral Dose	Fasting	Plasma Isotope Ratio 24 hr	48 hr	96 hr
		hr			
1	Milk	6	53 (53)	58 (56)	57 (57)
2	Milk	6	56	64	64
3	Bile salt	6	47 (44)	52 (51)	51 (52)
4	Bile salt	6	46	49	45
5	Bile salt	6	48	52	51
6	Bile salt	0	50 (48)	53 (53)	52 (54)
7	Bile salt	0	34	44	41
8	Bile salt	0	50	66	63

Rats weighed 260–358 g. They were 18 hr postabsorptive. The period of fasting refers to length of fasting after administration of oral and intravenous doses of labeled cholesterol. The doses were prepared in skim milk or in triolein emulsified with bile salt (see Methods). Cholesterol contributed by skim milk and bile salt mixture was negligible compared with the single oral dose of cholesterol. Figures in parentheses are isotope ratios for whole blood.

[a] See Methods.

In contrast to our earlier findings (1), the plasma isotope ratios of the 24-hr plasma samples were nearly always lower than those of 48-hr or subsequent samples (Table 1). When similar data for 41 rats were compared, the 24-hr plasma isotope ratio was 14% less than the ratio for the 48-hr plasma sample. In these same animals there were no significant differences between the plasma isotope ratios at 48-hr and those of subsequent sampling periods. Whether or not the 24-hr plasma isotope ratio is below that of subsequent plasma samples appears to be related to the diet. For rats in which the absorption test was performed twice (see Table 4), the 24-hr plasma isotope ratios were significantly ($P < 0.01$) below the later values when a semisynthetic diet was fed, but not when rats were fed a commercial chow. To avoid this early instability of the plasma isotope ratio, we have calculated cholesterol absorption from a single plasma isotope ratio at 48 hr or later, or from the average of such ratios when multiple blood samples were taken.

TABLE 2. Effect of sulfaguanidine on cholesterol absorption

Treatment[a]	Absorption[b] (100 − Feces)	Plasma Isotope Ratio	Recovery[c]
	%	%	%
Control	70 ± 2	58 ± 2	88 ± 3
Sulfaguanidine	46 ± 4	34 ± 1	88 ± 5

Rats weighed 285–300 g. They were 18 hr postabsorptive at the time of isotope dosage but had access to food immediately thereafter. The oral dose was prepared as described in the Methods section except that one-half the amount of bile salts was used. Feces were collected in tail cups.

[a] There were four animals in each group.

[b] The percentage of orally administered cholesterol absorbed was calculated either from the 4-day fecal excretion of orally administered labeled cholesterol or from a plasma isotope ratio (average of 48- and 96-hr values ± SD).

[c] The percent recovery is the percentage of the oral dose in a 4-day pool of fecal neutral sterols plus the percentage of the oral dose absorbed (calculated from the plasma isotope ratio).

TABLE 3. Effect of coprophagy on cholesterol absorption

Rat	Absorption (100 − Feces)	Plasma Isotope Ratio	Recovery
	%	%	%
		With Tail Cups	
1	81	56	75
2	67	62	95
3	68	52	84
4	64	61	97
5	57	50	93
6	51	57	106
7	61	72	111
8	55	49	94
9	50	54	104
Avg ± SD	62 ± 10	57 ± 7	95 ± 11
		Without Tail Cups	
10	62	48	86
11	42	37	95
12	65	60	95
13	60	57	97
14	35	42	107
15	44	50	106
16	69	71	102
17	59	54	95
18	48	56	108
19	67	81	114
Avg ± SD	55 ± 12	56 ± 13	101 ± 8

Rats weighed 156–186 g. Those listed in the top portion were provided with tail cups and those in the bottom portion were allowed to practice coprophagy. Rats 1–3 and 10–13 received 4 mg of cholesterol orally per 100 g of body weight, and the others were given 2 mg/100 g. The former also received twice as much triolein and bile salts in the oral dose as the latter. All animals were fed the semisynthetic diet. Column headings are the same as in Table 2.

Effect of sulfaguanidine

In another experiment the validity of the dual-isotope plasma ratio method was tested in animals that were receiving 1% sulfaguanidine in their diet. This drug has been shown to diminish cholesterol absorption (18). According to the data in **Table 2,** cholesterol absorption calculated either from the plasma isotope ratio or from fecal excretion data was decreased in sulfaguanidine-fed rats. The plasma isotope ratio showed an average decrease of 41% in cholesterol absorption, whereas the fecal excretion method indicated a 34% decrease. It should be noted that this decrease was observed in animals that were fasted 18 hr before cholesterol dosage but were given access to sulfaguanidine-containing food immediately after administration of the isotope doses. The recovery of the orally administered cholesterol dose, i.e., the sum of the isotope fraction recovered in a 4-day fecal pool and the fraction

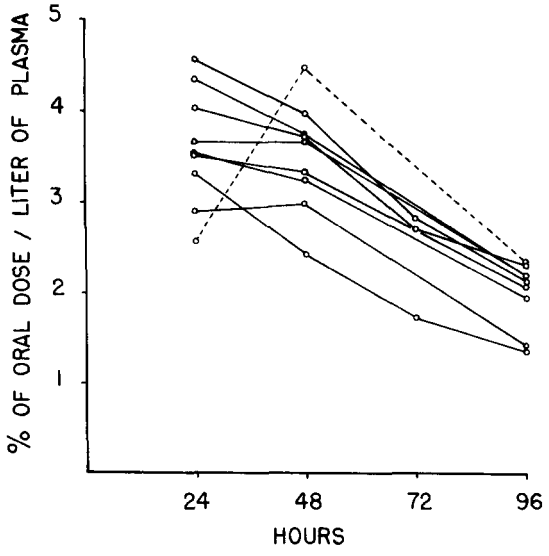

Fig. 1. Plasma isotope concentration curves after a single oral dose of labeled cholesterol in rats with tail cups (Table 3). The curve (- - -) representing animal 7 (Table 3) is exceptional in that maximal isotope concentration in plasma appears to be delayed.

absorbed (from plasma ratios), was 88% for both groups. This is somewhat lower than that seen previously (1).

Effects of coprophagy

In experiments reported previously (1) and in Tables 1 and 2, rats were fitted with tail cups that effectively prevent coprophagy (10). For the sake of simplicity it was of interest to know whether or not coprophagy materially altered the plasma isotope ratio of orally to intravenously administered cholesterol. **Table 3** shows that the average plasma isotope ratio was 57 in animals fitted with and 56 in rats without tail cups. Thus, it appears that prevention of coprophagy does not significantly affect absorption of an oral dose of cholesterol. The average recovery of orally administered isotope, calculated as before, was close to 100% in both groups.

When tail cups were not used, feces were collected in pans placed under the wire cage bottoms. Although the average values for cholesterol absorption calculated from the plasma isotope ratios agree very well with those obtained from fecal collections in both groups of animals, this agreement is only moderately good for individual animals (Table 3). From this experiment it is difficult to judge whether the plasma isotope ratios or the fecal excretion data give the more reliable information. However, animals 7 and 19, in which the plasma isotope ratio was about 12% higher than the absorption calculated from fecal excretion data, showed "abnormal" cholesterol absorption curves in that the 48-hr concentration of the orally administered isotope concentration was much greater than the 24-hr value (see curve for animal 7 in **Fig. 1**). This suggests that in animals in which absorption is greatly delayed the plasma isotope ratio overestimates cholesterol absorption. On the other hand, fecal excretion data may overestimate absorption when recovery of radioactive excretion products is incomplete. This appears to be the case for rat 1, and possibly for 3 and 10, which showed relatively low fecal sterol excretion and low recovery of radioactivity (last column, Table 3).

Repeatability of plasma isotope ratio

Another aspect of reliability of an absorption method is its repeatability in the same animal. We therefore gave an intravenous and an oral isotope dose to each animal (period 1, **Table 4**) and repeated this procedure 5 days later (period 2, Table 4). Plasma ratios were measured on the third day after each dosing. The studies shown in Table 4 were carried out in six rats maintained on the semisynthetic diet and six fed commercial rat chow. In general, the agreement between the plasma isotope ratios obtained from the first and second periods was good. Animal 10 appears to be an exception (33 vs. 73% absorption), but absorption calculated from fecal excretion data shows the same difference for the two test periods. It is therefore likely that for animal 10 the disagreement between the absorption values obtained by both tests in the two periods represents a difference of biological origin and not a methodological difficulty.

TABLE 4. Repeatability of cholesterol absorption methods

		Absorption				
		Period 1		Period 2		
Rat	Diet[a]	Plasma Isotope Ratio	(100 − Feces)	Plasma Isotope Ratio	(100 − Feces)	Area Ratio
		%	%	%	%	%
1	SS	50	48	52	71	51
2	SS	47	50	53	74	52
3	SS	48	50	53	72	53
4	SS	58	63	64	74	60
5	SS	41	45	34	49	36
6	SS	50	48	56	74	55
7	Ch	52	58	49	62	53
8	Ch	35	40	37	46	40
9	Ch	38	40	49	47	52
10	Ch	33	38	73	67	73
11	Ch	45	44	50	52	51
12	Ch	36	43	51	57	50

Animals weighed 188–221 g. They were dosed with [^{3}H]cholesterol orally and [^{14}C]cholesterol intravenously on two occasions 5 days apart. The second dose of radioactivity was approximately 20 times greater than the first, but the amounts of cholesterol, bile acid, etc., were the same. Cholesterol absorption was calculated from the plasma isotope ratio on the third day after each dosage (columns 3 and 5), from 4-day fecal pools (columns 4 and 6), and from the ratio of the areas under the ^{3}H and ^{14}C plasma concentration curves generated during the 21 days after the second dosing (column 7). All animals were fasted 18 hr before each dose and 6 hr thereafter. Column headings are the same as in Table 2.

[a] SS, semisynthetic diet gelled with 1.3% agar; Ch, Agway rat and mouse chow.

TABLE 5. Effect of intravenous dose on dual-isotope absorption test

Rat	Intravenous Dose	Absorption (100 − Feces)	Absorption Plasma Isotope Ratio	Recovery
		%	%	%
1	Colloid	50	46	96
2	Colloid	60	57	97
3	Colloid	56	53	97
4	Colloid	57	60	103
Avg ± SD		56 ± 4	54 ± 6	98 ± 3
5	Tween	59	72	113
6	Tween	56	66	110
7	Tween	52	52	100
8	Tween	48	55	107
9	Tween	61	88	127
10	Tween	64	74	110
Avg ± SD		57 ± 6	68 ± 13[a]	111 ± 9

Rats weighed 142–176 g. Column headings are the same as in Table 2. Plasma ratios were calculated from the average of 48- and 96-hr values.

[a] This value differs significantly ($P < 0.05$) from the values for all colloid-injected animals in this table and animals 10–19 in Table 3.

In the animals fed the commercial chow regimen, the fecal excretion data in periods 1 and 2 showed no consistent differences, whereas in the animals fed the semisynthetic diet, the fecal excretion of orally administered isotope was consistently less in the second test. It would appear, therefore, that in this experiment the dual-isotope plasma ratio test showed better repeatability than the fecal excretion method.

As discussed in the Methods section, a third measure of cholesterol absorption may be obtained from the ratio of the areas under the plasma isotope concentration curves for the oral and the intravenous doses. Cholesterol absorption calculated from the area ratio method, when the areas are extrapolated to infinite time, is independent of delays in the absorption of the oral dose or release of the colloid from the liver. In our experiments the area was available up to day 21, which, according to data presented in the following section, represents approximately 65% of the area under the curve extrapolated to infinity. A time lag of 0.5 day between the appearance of label from the oral and the intravenous doses in the plasma would only produce a 1.5% error in the area ratio method when measured up to day 21. This contrasts with a potential error of 29% in the 48-hr plasma ratio and of 12% in the 96-hr plasma ratio if a time lag of 0.5 day existed between the oral and intravenous doses. The excellent agreement, shown in Table 4, between the cholesterol absorption calculated by the area ratio method and the plasma isotope ratio at a single (72 hr) point shows that there is no significant time lag between the appearance of the oral and intravenously administered isotope doses. In addition, it supports the use of the plasma isotope ratio at a single time point under the conditions of this experiment.

Effect of intravenous dose

Thus far all experiments were done with an intravenous dose of colloidal cholesterol in the belief that the rapid removal of the colloid by the Kupffer cells of the liver (2, 3) and the gradual release of label back into the circulation was more similar to the appearance of orally administered cholesterol than a dose that would manifest maximal radioactivity at the time of injection. However, an experimental test of this principle had not been made. We therefore injected intravenously labeled colloidal and Tween 20-solubilized cholesterol into four rats (**Fig. 2,** *left*). The plasma samples taken at 6 and 12 hr after dosing show an ascending curve for colloid being released from the liver and a steeply descending curve for the dose in Tween 20. To prevent interaction of the injected Tween 20 with the colloid dose, we injected four rats intravenously with labeled colloidal cholesterol and six rats with labeled cholesterol that had been solubilized with Tween (7). From Fig. 2, *right,* it is evident that, 24 hr or more after dosage, the concentration of Tween-solubilized labeled cholesterol was significantly lower than that of cholesterol administered in the colloidal form. It is likely that the Tween-solubilized material equilibrates more rapidly with the exchangeable cholesterol pools in the body, and therefore its degradation and excretion begin somewhat sooner than that of the colloidal material. This difference is reflected in a difference in the plasma ratios of orally and intravenously administered doses, as shown in **Table 5.** In the animals receiving the colloidal cholesterol as an intravenous dose, the average plasma ratio is 54%, whereas for the animals receiving cholesterol solubilized in Tween, this ratio averages 68%. The latter appears too high as an index of cholesterol absorption, as

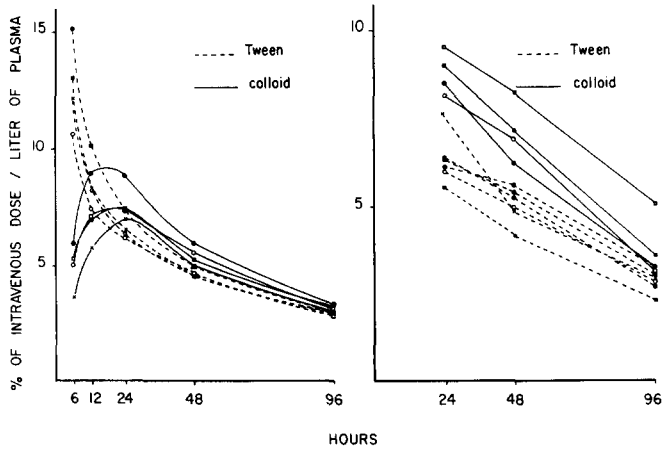

Fig. 2. Disappearance of labeled colloidal cholesterol and Tween 20-solubilized cholesterol. *Left,* animals received the two intravenous doses; *right,* animals were injected either with colloid or with Tween 20-solubilized labeled cholesterol.

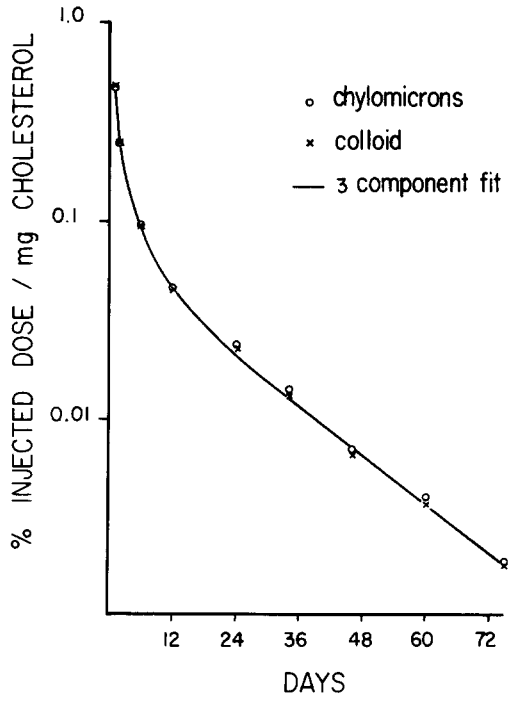

Fig. 3. Disappearance of colloidal [^{3}H]cholesterol and [^{14}C]cholesterol-labeled chylomicrons from plasma of a rat (rat 8, Table 5).

shown by the excessive "recovery" of label in five out of six animals when the cholesterol absorbed and excreted are added. When the average plasma ratio for the 6 Tween-injected animals is compared with that for the 14 colloid-injected animals (Tables 3 and 5), which were kept on the same diet and were without tail cups, the difference is significant at the 5% level.

Comparison of colloidal cholesterol with chylomicrons

One further test concerning the validity of the dual-isotope plasma ratio absorption test is a comparison of the metabolism of colloidal cholesterol with that of cholesterol contained in chylomicrons. For this purpose, eight rats were injected with labeled colloidal cholesterol and then infused with rat lymph chylomicrons containing a different cholesterol label. A typical pair of disappearance curves is shown in **Fig. 3.** In some animals the curves generated by the colloid dose differed very slightly from those obtained with chylomicrons; in other animals there was no detectable difference. The average plasma ratio of isotope derived from chylomicrons and colloid of eight animals in which the ratio of the injected doses was unity was 1.05 for day 1 and either 0.99 or 1.00 for all subsequent sampling periods.

Table 6 shows the plasma cholesterol concentrations, the production rates calculated from the chylomicron and colloid disappearance curves, and excretion rates of endogenous sterol as determined from the total radioactive sterol in the feces and the specific activity of the plasma cholesterol. The excretion rates were calculated only from injected colloid, because the fecal radioactivity derived from chylomicrons was insufficient. The production rates calculated from the plasma disappearance curves and fecal excretion rates were within 6% in two animals but differed by 22 and 23% in the other two.

DISCUSSION

The literature on cholesterol absorption in the rat is not very extensive, and the values reported span an enormous range. Wells, Anderson, and Ma (19) reported that only 7.5% of labeled cholesterol was recovered in a 24-hr thoracic duct lymph collection. At the other extreme, Lutton and Chevallier (11) reported an average cholesterol absorption in intact rats as high as 97%. Several other studies gave values that lie in between: Bloomfield (20) and Borgström (15) reported approximately 50% cholesterol absorption in intact rats, and Sylvén and Borgström (21) found values of 40–45% in thoracic duct-cannulated rats. Some of these differences may be related to the dose of dietary cholesterol, to the presence of bile salts in the diet (11), to the effects of surgery in thoracic duct-cannulated animals (19), and to other methodological differences. It is of interest that in our study the percentage of cholesterol absorbed in the few rats that received double the amount of cholesterol in the oral dose (see Table 3) was not significantly lower than that for the other animals. However, the number of animals involved in this comparison is too small to furnish reliable information on this point. Borg-

TABLE 6. Cholesterol turnover in rats injected with colloid and with lymph labeled with cholesterol

Rat No.	Rat Wt.	Plasma Cholesterol	Production Rate		Fecal Excretion, Colloid
			Colloid	Chylomicrons	
	g	*mg%*	*mg/day*	*mg/day*	*mg/day*
1	425	51	44	44	
2	415	62	31	30	
3	360	51	29	27	
4	350	54	26	25	
5	470	48	35	37	47
6	395	50	25	26	27
7	370	56	29	26	35
8	500	74	42	41	39

Rats were injected with labeled colloidal cholesterol and then infused for 3.5 hr with cholesterol-labeled lymph. Simultaneous disappearance of ^{14}C and ^{3}H was measured for 60–75 days. Animal weights given were at the beginning of the study; the rats were fed Agway rat and mouse chow. Production rates were calculated from areas under the plasma cholesterol specific activity curves. Fecal excretion of endogenous cholesterol, measured only for animals 5–8, was calculated from the daily total fecal radioactivity derived from the colloid dose divided by the daily specific activity of plasma cholesterol. The values listed are averages for days 72–75.

ström (15) observed a gradual decrease in percentage of cholesterol absorbed with increasing cholesterol dose in thoracic duct-cannulated rats. Although in our studies the results reported are probably free from the effect of surgical intervention, one must recognize that the values for percentage of cholesterol absorbed apply to the type of emulsion used and do not necessarily represent values obtained for other forms of dietary cholesterol or other types of diets.

The dual-isotope plasma ratio method is based on the assumption that the absorbed portion of an orally administered dose is distributed and metabolized in the same manner as an intravenously administered dose. In the present series of experiments we have obtained further evidence to support this and other points related to the method. When colloidal cholesterol is administered simultaneously with cholesterol-labeled chylomicrons, the disappearance curves from plasma of labeled cholesterol from both sources were the same within the errors of measurement. Consequently, production rates of cholesterol calculated from the two isotope doses agreed closely. When the intravenous colloidal cholesterol dose was compared with a Tween 20-solubilized cholesterol preparation, a consistent difference between the disappearance curves was observed. Moreover, when Tween 20-solubilized cholesterol was used to calculate cholesterol absorption in animals that had also been given an oral dose of cholesterol, the calculated recovery of the oral dose, i.e., the sum of absorbed and excreted cholesterol, exceeded 100%. This probably means that when Tween 20 was used to solubilize the intravenously administered cholesterol, the plasma isotope ratio overestimated cholesterol absorption.

In our earlier publication we showed that the plasma isotope ratio of oral and intravenously administered cholesterol remained constant beyond 24 hr after isotope dosage. For most animals in the present study, the 24-hr plasma isotope ratio appeared to be lower than that at 48 hr or later. We therefore have used only plasma ratios obtained at 48 hr or later for the measurement of cholesterol absorption. In a number of comparisons we have shown that in the same animal these ratios were more reproducible than cholesterol absorption determined from fecal samples but that on the average they gave results similar to those obtained from fecal data. The plasma isotope ratio does not appear to depend significantly on the practice or absence of coprophagy in the animals. This is somewhat surprising because, according to Lutton and Chevallier (22), rats reingest 41% of the excreted fresh feces. An appreciable amount of the reingested sterol is in the form of coprostanol, which is not readily absorbed, but one would have expected that some of the unabsorbed labeled cholesterol would have been absorbed on the second passage through the alimentary canal.

In the present experiments we have tested the plasma isotope ratio method in animals maintained on high and low residue diets (Table 4). Although animals on these diets show differences in intestinal transit time and bile acid excretion rates (23, 24), the plasma ratio method agrees equally well with absorption calculated from fecal excretion data in both groups of animals. In other experiments, animals fed 1% sulfaguanidine in the diet showed similar decreases in cholesterol absorption when measured by the plasma ratio procedure or by the fecal excretion method.

It would appear therefore that, in the rat, the dual-isotope plasma ratio method is at least as reliable for the measurement of cholesterol absorption as methods based on the analysis of feces. The plasma ratio method has the advantage of being technically simpler and independent of possible sterol destruction in the lower part of the gastrointestinal tract.

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