Evaluation of an isotope ratio method for measurement of cholesterol absorption in man

Paul Samuel,[1] **John R. Crouse,**[2] **and E. H. Ahrens, Jr.**

The Rockefeller University, New York, NY 10021

Abstract Recently an isotope ratio method (IRM) was developed for measuring cholesterol absorption in rats by analysis of radioactivity in peripheral blood (Zilversmit, D. B. 1972. *Proc. Soc. Exp. Biol. Med.* **140:** 862–865). To validate it in man we have compared cholesterol absorption by a fecal radioactivity method (FRM) with that simultaneously measured by IRM in 14 patients (15 experiments) hospitalized on a metabolic ward. Cholesterol absorption by FRM was assayed as fecal recovery of orally administered [^{14}C]cholesterol, after correction with markers for fecal flow (chromic oxide) and cholesterol degradation (β-sitosterol). Simultaneously, [^{3}H]cholesterol was administered intravenously, and the dose-normalized ratio of [^{14}C]- to [^{3}H]cholesterol was repeatedly assayed in plasma. After 72 hours the ratio became constant in each patient and remained so for as long as 63 weeks (five additional outpatient studies). In three patients the fecal data were unsatisfactory because of poor recoveries of chromic oxide and radioactive cholesterol. In the remaining 11 patients (12 experiments) the mean cholesterol absorption by IRM was 42.1% (range 15.7–62.9%) and by FRM 36.6% (range 13.8–58.8%). There was good to excellent agreement between the two methods in the same patient, except in one experiment. Statistical analysis of these 12 comparisons by estimating confidence intervals showed that we can be 95% confident that the two absorption methods will produce results within 5 percentage points, and 99% confident that the differences are less than 7 percentage points. Although we conclude that IRM affords results that are concordant with those obtainable by earlier validated methods, we urge that its suitability for outpatient studies be further examined in more extensive trials.

Supplementary key words sterol balance · sterol degradation · chromic oxide recovery · fecal methods · plasma method · intravenous tracer · oral tracer

In the recent past, four methods for measuring cholesterol absorption in man were evaluated in this laboratory (1). What was required in all of these procedures was hospitalization of patients on a metabolic ward, eucaloric intake of a constant dietary mixture, administration of radioactive cholesterol, attainment of a metabolic steady state, collection of feces for varying lengths of time, and analysis of fecal neutral steroid radioactivity. It developed that the simplest and least time-consuming of the four methods was the measurement of nonabsorbed cholesterol appearing in the feces over a 6–8 day period after oral administration of labeled cholesterol; appropriate corrections for sterol degradation during its transit through the intestinal canal were required. This method, first described in 1968 by Borgström (2), was validated in this laboratory in 1971 by comparison with three previously published methods and was designated Method IV (1); confirmatory results have subsequently been reported by Sodhi et al. (3) who have modified the method somewhat without serious loss in precision. A fifth method, separately described by us in 1971 (4), is applicable in situations of very rapid cholesterol synthesis when intravenously administered labeled cholesterol fails to reach rapid equilibrium with endogenously synthesized cholesterol.

Recently, Zilversmit proposed a method for measuring cholesterol absorption (5, 6) that involved only the analysis of plasma radioactivity and did not require stool collections. In principle it is similar to a procedure for measurement of drug absorption that has been used by clinical pharmacologists for at least 40 years (7). What is required is the simultaneous administration of ^{14}C- and ^{3}H-labeled cholesterol, one orally and the other intravenously, and subsequent comparison of the two plasma cholesterol specific activity curves. Under these conditions, if the absorption of the oral dose is 100%, the oral and intravenous curves will be superimposed; in contrast, if the absorption of the oral dose is zero, radioactivity from that source will fail to appear in the plasma. In most cases the oral curve falls between these two extremes, and percent absorption can be calculated as the ratio of the two plasma radioactivities (oral to

Abbreviation: SA, specific activity.

[1] Address reprint requests to Dr. Paul Samuel, The Rockefeller University, New York, N.Y. 10021.

[2] Dr. Crouse was a New York Heart Association Fellow during the period of this study. His present address is: Department of Medicine, V. A. Hospital, La Jolla, California 92161.

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TABLE 1. Relevant clinical data in 14 patients in whom cholesterol absorption was tested simultaneously by two methods

Patient	Age	Sex	Height	Weight	% Ideal Weight[a]	Diagnosis[b]
	yr		*cm*	*kg*		
1 J.L.	52	M	159	63	117	Hyperglyceridemia, IHD
2 S.B.	52	M	171	86	121	Hyperglyceridemia
3 N.A.	56	F	158	55	100	Hypercholesterolemia, IHD, PVI
4 M.N.	65	F	162	53	102	Essential hypertension, HHD
5 S.W.	44	M	173	68	104	IHD
6 A.R.	62	F	162	63	108	Hypercholesterolemia, PVI
7 R.C.	41	M	179	106	138	Hyperglyceridemia, IHD
8 L.M.	34	M	169	79	124	Hypercholesterolemia
9 J.S.	74	M	174	73	104	IHD
10 E.G.	57	M	166	63	106	Combined hyperlipidemia, IHD
11 A.H.	55	F	152	62	115	IHD
12 E.G.	50	F	162	106	168	Diabetes mellitus (mild)
13 S.H.	47	M	176	75	112	Hyperglyceridemia, gout, Peyronie's disease
14 E.C.	58	F	155	73	125	Hyperglyceridemia

[a] Percent of ideal weight according to life insurance tables (10).
[b] Abbreviations: IHD, ischemic heart disease; PVI, peripheral vascular insufficiency; HHD, hypertensive heart disease.

intravenous) after dose normalization. The isotope ratio method has been found to be a valid measurement of cholesterol absorption in the rat (5, 6) and in primates (8, 9) but not in the rabbit.

There are two basic requirements of this method: (*a*) there can be no isotope effects in the body's handling of the two radioactive compounds, and (*b*) time must be allowed for the two downward slopes to become entirely parallel before the two radioactivities are compared. The present report examines these two requirements in experiments carried out in 19 patients over the past 3 years; we could obtain no evidence for the occurrence of isotope effects, and found that in all cases the peak of radioactivity of the oral dose was reached in 3 days or less and that the curves remained parallel thereafter. On the basis of these findings we compared absorption results by Method IV and by the isotope ratio method in 14 patients with normal bowel function; we found we could be 95% confident that the difference in absorption data obtained by the two methods was less than 5 percentage points (±).

This report also describes a simplified modification of Zilversmit's method (5) and notes several areas for future study that must be explored before the isotope ratio method is considered suitable for wide application in human studies.

METHODS

Patients

Studies were carried out on 14 patients during hospitalization for 2.5–36 weeks on a metabolic ward. Their ages, sex, body builds, and clinical diagnoses are listed in **Table 1**; plasma lipid levels and dietary intakes are given in **Table 2**. There were eight men and six women aged 34–74 years. Nine patients had elevated plasma lipids, and seven had ischemic heart disease. One patient had mild diabetes mellitus without glycosuria and required no drug or insulin therapy. Patients 8, 12, and 13 were taking clofibrate (500 mg four times per day) throughout the study; clofibrate has little or no effect on cholesterol absorption, according to previous studies (11).

Five other patients (No. 15–19) were studied as outpatients. Two were male and three were female; their ages were 49–72; clinical diagnoses included ischemic heart disease in three and essential hypertension in

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TABLE 2. Dietary intakes and plasma lipid levels[a]

Patient	Diet	K-calories[b]	Dietary Sterols		Plasma Lipids	
			Cholesterol	β-Sitosterol	Cholesterol	Triglycerides
			mg/day		*mg/dl*	
1	Formula[c]	2300	547	331	255 ± 14 (33)[d]	230 ± 33 (22)[d]
2	Formula[c]	3030	721	436	253 ± 9 (38)	520 ± 170 (30)
3	Solid[e]	1800	533	182	535 ± 25 (10)	165 ± 35 (10)
4	Solid	1900	270	130	265 ± 14 (19)	134 ± 20 (19)
5	Solid	2100	129	280	253 ± 17 (12)	174 ± 14 (12)
6	Solid	2000	429	143	431 ± 21 (16)	150 ± 19 (16)
7	Solid	2700	521	347	256 ± 15 (12)	424 ± 182 (12)
8	Solid	2700	128	356	371 ± 9 (10)	147 ± 21 (10)
9	Solid	2150	122	174	183 ± 17 (22)	98 ± 12 (22)
10	Solid	2200	104	190	357 ± 46 (12)	568 ± 68 (12)
11	Solid	1800	75	247	238 ± 16 (17)	83 ± 13 (17)
12	Solid	3500	605	228	244 ± 9 (14)	170 ± 40 (14)
13	Solid	1900	209	219	184 ± 24 (10)	244 ± 104 (10)
14	Solid	2400	96	359	235 ± 9 (11)	267 ± 47 (11)

[a] During metabolic steady state.
[b] Daily energy intake required to maintain constant body weight, in kilocalories (1 kilocalorie = 4.184 kilojoules).
[c] Oral formula feeding (12, 13) with lard equal to 40% of total calories.
[d] Numbers in parentheses are numbers of determinations.
[e] Solid food diets given repetitively in 2- or 3-day cycles, all found to furnish the same mixture of major nutrients, cholesterol, and β-sitosterol each day. Fat content equals 30 or 40% of total calories.

two patients. Two were normolipidemic and three had hypercholesterolemia. None of the patients had bowel disease and all were eating ad libitum.

Diets

In Patients 1 and 2 body weights were maintained constant by liquid formula feedings administered orally as previously described (12, 13); dietary fat contributed 40%, protein 15%, and carbohydrate 45% of total energy intake. The formula contained 119 mg of cholesterol and 72 mg of β-sitosterol per 500 kcal, and the fat that was fed (lard) was similar in quality to that occurring in the average American diet. Minerals and vitamins were supplemented as described previously (12, 13).

In the remaining 12 patients body weights were maintained constant by the feeding of a rotating menu of solid foods in 2- or 3-day repetitive cycles. The percentage of energy derived from the major nutrients was calculated from standard food tables and was held constant from one day's menu to the next; fat contents were from 30 to 40% of total energy. Sterol contents of each day's food intake were analyzed by standard methods of extraction and analysis by gas–

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liquid chromatography (14). Daily cholesterol intakes (Table 2) in all patients varied from 75 to 721 mg; β-sitosterol contents were from 130 to 436 mg per day. The energy intakes required to maintain each patient at constant weight throughout the study periods are shown in Table 2.

Radioactive sterols

[1,2-^{3}H]Cholesterol and [4-^{14}C]cholesterol were obtained from New England Nuclear Corp., Boston, MA; they were purified by thin-layer chromatography on Florisil (Floridin Co., Tallahassee, FL) with ethyl ether–heptane 45:55 (v/v). Only that material that chromatographed with the same R_f value as a pure cholesterol standard was administered to patients. For intravenous administration, labeled cholesterol dissolved in 1 ml of ethanol was suspended in 150 ml of saline and immediately infused intravenously. Residual radioactivity in the infusion set was determined after ethanol extraction in order to determine the exact dose administered. For oral administration, labeled cholesterol in ethanol was mixed with liquid formula or whole milk and was given at 8:30 AM. After drinking this mixture, patients rinsed the glass with about 50 ml of the suspending fluid and drank it; this rinse was repeated. All radioactivity remaining in the glass was measured by ethanol extraction and the net amount actually administered to the patients was determined. Doses of radioactivity varied from 2 to 79 μCi; for technical reasons in counting, oral and intravenous doses were kept approximately the same. Lately, to minimize dose levels without sacrificing technical precision in measuring isotope ratios within the first week, we have given the ^{3}H dose intravenously and the ^{14}C dose orally, about 2 μCi each. Radioactivity was measured in a Packard Tri-Carb scintillation counter (Model 3380-3390, Packard Instrument Co., Inc., Downers Grove, IL) with quench corrections performed automatically by an absolute activity analyzer (Packard Instruments, Model 544), as previously described (14).

Clinical procedures

After an overnight fast, patients were given an intravenous infusion of labeled cholesterol as described above, immediately followed by an oral dose of cholesterol containing a different isotope. Five to 35 min after the oral dose of radioactivity, a breakfast containing fatty food (milk, margarine) was served.

Analytical methods

Plasma lipids. Concentrations of plasma cholesterol and triglycerides were determined twice weekly by the methods of Block, Jarrett, and Levine (15) and Kessler and Lederer (16), respectively, on the Auto Analyzer (Technicon Instruments Corp., model II, Tarrytown, NY).

Fecal neutral steroids were isolated from 24-hr stool collections; their mass and radioactivity were measured by methods developed in this laboratory (14). Dietary β-sitosterol was used as an internal standard to correct for losses of cholesterol during intestinal transit (17). Chromic oxide was employed as an internal standard to correct for fecal flow variations and stool recovery (18).

Calculations

Cholesterol absorption was calculated by the fecal radioactivity method (Method IV) (1) as:

> Percentage of dietary cholesterol absorbed = [1 − (radioactivity in fecal cholesterol ÷ fecal β-sitosterol) × (daily dietary β-sitosterol ÷ radioactivity in administered cholesterol)] × 100

Method IV results were also corrected for the small amount of orally administered labeled cholesterol that had been absorbed and subsequently excreted into the intestinal contents. This was done by determining the percent of the labeled cholesterol given intravenously that was excreted in feces over the time period of the stool collections, and subtracting this percentage from the percentage of orally administered cholesterol found in that stool collection. The delay of the peak of oral isotope in the plasma was taken into consideration in these calculations. This correction could not be carried out in Patients 1–4 because they had received labeled β-sitosterol in the test meal; however, in Patients 5–14 it was technically feasible to make this calculation.

Calculations for absorption by the isotope ratio method were performed on the two cholesterol specific activity (SA) decay curves on successive days, each expressed in terms of percent dose per g of plasma cholesterol in order to normalize the differences in doses actually administered. Data points were curve-fitted by a standard nonlinear least-square fit, and t ½ values were determined by computer. After the two curves became parallel, cholesterol absorption was calculated:

$$\%\ \text{absorption} = \frac{\text{SA of cholesterol given orally}}{\text{SA of cholesterol given i.v.}} \times 100$$

The number of pairs of data points collected in the different patients varied from 5 to 8. Absorption results for each patient were expressed as the mean ±SD for results obtained with these pairs.

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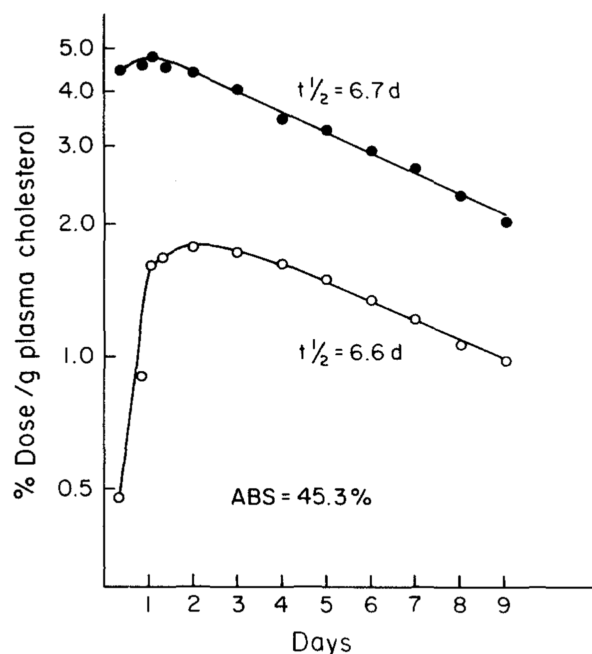

Fig. 1. Specific activity–time course curves of plasma cholesterol from 0–9 days after i.v. administration of [4-^{14}C]cholesterol and oral administration of [1,2-^{3}H]cholesterol in Patient 4. Note that the t ½ values were almost identical. The ^{14}C/^{3}H ratio (×100) became constant (45.3 ± 1.2) after 2 days in this study; in 15 studies this constancy was reached in 3 days or less.

Computer analyses and area integrations of the curves were carried out by Dr. Sidney Lieberman (Department of Mathematics, Queens College, CUNY). In addition, the area under each curve was integrated from zero time to infinity by extrapolating the first available datum to zero and the log-linear exponential decay curve to infinity, and percent absorption of cholesterol was then calculated by comparing the areas under each of the pairs of SA curves:

$$\% \text{ absorption} = \frac{\text{area under oral curve}}{\text{area under intravenous curve}} \times 100,$$

or

$$= \frac{\int_0^\infty w_{\text{PO}}(t)\,dt}{\int_0^\infty w_{\text{IV}}(t)\,dt} \times 100,$$

where w_{PO} refers to the SA curve of the orally administered and w_{IV} to the intravenously administered labeled cholesterol.

Modified plasma method

After it became clear that the two SA decay curves always became parallel 3 days or more after isotope administration, we found it advantageous in outpatient pilot studies to measure the plasma isotope ratio at a single time point on the third day (or later). This made it possible to schedule two visits to the clinic, one for dose administration and one for plasma isotope ratio measurement. To increase the precision of this measurement, a single blood sample was analyzed in sextuplicate. Although this modification does not allow a calculation of areas under the two curves, the accuracy of the results obtained was not impaired (see Results).

RESULTS

Parallelism of two plasma cholesterol specific activity curves

A critical requirement of the isotope ratio method is that the body metabolize the two radioisotopic cholesterols identically, i.e., with no isotope effects. Obviously, since the routes of entry of the two tracers are different, one oral and the other intravenous, it is not surprising that the time-course curves of the two plasma cholesterol specific activities are quite different; Nilsson and Zilversmit (19) have defined some of the causes of these differences during the first few hours after co-administration of the two isotopic sterols. We did not examine these early time points but sampled first at 4–8 hr (**Fig. 1**), and found that the peak of the i.v. cholesterol curve always preceded that of the oral cholesterol curve by about 24–48 hr. However, from that time onward, the two curves were

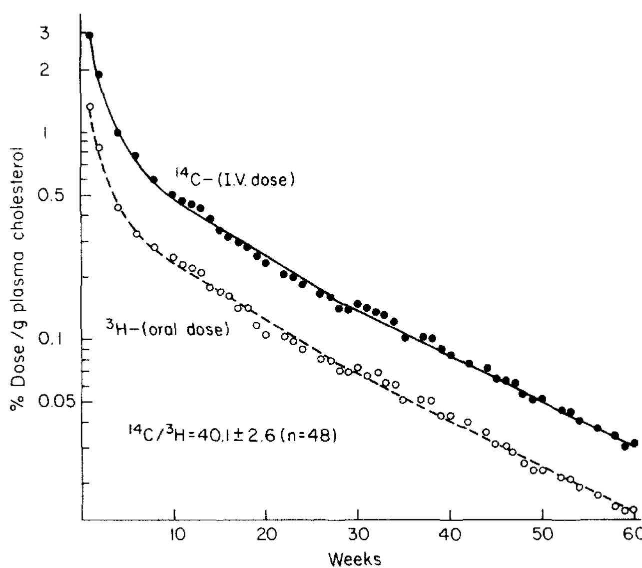

Fig. 2. Constant ratio of ^{14}C/^{3}H in plasma cholesterol from 2–60 weeks after co-administration of labeled cholesterols ([4-^{14}C] intravenously, [7α-^{3}H] orally) in Patient 6.

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strictly parallel (Fig. 1), even for very long time periods (**Fig. 2**). **Table 3** presents computer analyses of the three exponentials of the two radioactivity curves in five outpatients studied 1–63 weeks. The three *t* ½ values of the two curves were nearly identical in every patient, and the absorption data calculated at early, intermediate, and late time periods were almost indistinguishable.

In the 15 studies carried out in 14 inpatients on the metabolic ward, SA ratios were obtained daily for 6–8 days after co-administration of the two radioactive cholesterols and the peak of the oral cholesterol curve never occurred later than the third day. Thereafter the ratios remained constant (within experimental error), as indicated by the small standard deviations around the mean in each patient (**Table 4**).

Absorption data obtained by two methods

All fecal radioactivity and plasma isotope ratio results in the 14 patients studied under metabolic ward conditions are presented in Table 4.

(*a*) *Chromic Oxide and β-sitosterol recoveries.* In 12 technically acceptable experiments chromic oxide recoveries exceeded 80%, with the mean excretion for the group being 97.2%. However, in Patients 12–14 only 75–79% of administered chromic oxide was recovered, and the total recovery of labeled cholesterol (see below) was only 83–90%, mean 87.3%. We have previously described this nonideal behavior that occurs in a small percentage of patients (18) and have presented our reasons for discarding any experimental results obtained in such circumstances. Accordingly, Patients 12, 13, and 14 are listed separately in Table 4, and the results of absorption in these three patients were excluded from our statistical comparison of the two methods. Patient 11 excreted more than 100% of the chromic oxide during the 7 days of stool collection for the two absorption tests performed (147 and 111%). However, in this patient, the mean chromic oxide recovery over a 36-week hospitalization period was exactly 100% (see Table 4, footnote *j*), and so we have considered that the two studies in Patient 11 were technically acceptable.

The only patients who were maintained solely on formula feedings were Patients 1 and 2. As often seen in formula-fed patients, the recovery of the β-sitosterol marker was low (56 and 54%, corrected for chromic oxide recovery to 62 and 60%), due to sterol degradation during the course of intestinal transit (17). The remaining 10 studies were carried out in patients maintained on solid food diets; as expected (21, 22), there was much less sterol degradation, with β-sitosterol recoveries averaging 90%. However, three of these patients maintained on solid foods had β-sitosterol recoveries of 55% (Patient 5), 73% (Patient 9) and 75% (Patient 6). These three studies (out of nine) represent important exceptions to the commonly held belief (21, 22) that degradation of neutral steroids in patients eating regular solid-food diets is negligible: accordingly, it is the standard practice in this laboratory to measure β-sitosterol recoveries routinely in all such studies and to apply the correction factors thus obtained in every case.

(*b*) *Fecal radioactivity data.* The mean absorption by

TABLE 3. Lengths and half-lives of 1st, 2nd, and 3rd exponentials of plasma cholesterol specific activity–time curves, and cholesterol absorption calculated on the separate exponentials, after simultaneous administration of [^{14}C]cholesterol (i.v.) and [^{3}H]cholesterol (orally)

Patient	Length of Exponential[a]			[^{14}C]Cholesterol Curve[b]			[^{3}H]Cholesterol Curve[b]			Cholesterol Absorption[c] Exponential		
	First	Second	Third	t ½$_1$	t ½$_2$	t ½$_3$	t ½$_1$	t ½$_2$	t ½$_3$	First	Second	Third
		weeks			*days*			*days*			*% (Mean ± SD)*	
15 J.L.	1–8	9–35	36–63	5.0	24	88	2.0	19	82	49.5 ± 3.8 (8)	49.9 ± 2.1 (22)	48.9 ± 2.1 (19)
16 S.K.	1–7	8–20	21–60	3.6	20	94	2.4	28	88	49.3 ± 2.3 (5)	48.6 ± 2.0 (12)	49.3 ± 3.4 (31)
17 S.P.	1–8	9–28	29–57	2.0	18	97	2.9	15	92	34.2 ± 1.5 (9)	34.0 ± 2.1 (19)	33.2 ± 1.2 (27)
18 N.S.	1–9	9–58		12	82		14	78		39.8 ± 1.5 (9)	39.5 ± 2.9 (38)	
19 D.B.	1–8	9–29	30–55	5.9	23	79	5.5	19	79	42.5 ± 2.6 (7)	41.2 ± 2.7 (17)	41.0 ± 1.9 (21)

[a] In all patients the earliest data points were obtained 1 week after co-administration of labeled cholesterol orally and intravenously.
[b] Curves of 55–63 weeks' duration were analyzed by computer (20); t 1/2$_1$ signifies half-life of first exponential, etc.
[c] Mean (±SD) of the isotope ratios of corresponding data points on each exponential. Numbers in parentheses are numbers of analyses of plasma cholesterol isotope ratio.

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TABLE 4. Comparison of cholesterol absorption data obtained by two methods

		Recovery of Internal Standards (%)			Fecal Radioactivity Method				Plasma Radioactivity Method		
			β-Sitosterol		% Dose Recovered in Feces		% Absorption		% Absorption		
Patient	Duration of Study[a]	Cr_2O_3	Uncorr.	Corr.[b]	Uncorr.[c]	Double-corr.[d]	Double-corr.[e]	Triple-corr.[f]	Isotope Ratio[g]	Area Ratio[h]	Total Recovery[i]
	weeks				Technically acceptable studies				*mean ± SD*		%
1	32	90	56	62	30.6	54.8	45.2		42.1 ± 1.2	43.3	96.9
2	18	90	54	60	27.5	51.0	49.0		57.2 ± 1.7	49.2	108.2
3	2.5	98	99	101	54.8	55.9	44.1		49.2 ± 1.2	49.5	105.1
4	5	93	108	113	45.7	49.1	50.9		45.3 ± 1.2	42.0	94.4
5	3	86	47	55	40.8	86.2	13.8	15.2	15.7 ± 0.6	14.9	101.9
6	3	89	67	75	27.5	41.2	58.8	60.3	62.9 ± 2.5	67.7	104.1
7	3	83	86	104	60.5	72.9	27.1	33.8	36.0 ± 0.7	34.6	108.9
8	2.5	95	83	87	67.0	81.0	19.0	21.5	44.3 ± 0.5	43.3	125.3
9	15	94	69	73	48.1	70.1	29.9	30.7	37.6 ± 1.4	36.4	107.7
10	4	90	84	93	55.0	65.7	34.3	35.7	44.6 ± 0.1	44.9	110.3
11A	36	147[j]	148[j]	100	58.2	58.2	41.8	43.2	41.8 ± 0.9	40.5	100.0
11B	36	111[j]	110[j]	100	74.9	74.9	25.1	26.5	29.0 ± 0.4	28.6	103.9
Mean		97.2					36.6		42.1	41.2	105.6
					Technically unacceptable studies						
12	3	78	59	75	34.2	58.3	41.7		24.8 ± 1.2	23.0	83.1
13	2.5	75	96	128	55.7	74.3	25.7	26.4	15.7 ± 0.4	15.6	90.0
14	16	79	77	98	35.8	46.2	53.8	56.2	42.6 ± 0.4	41.3	88.8

[a] Duration of hospitalization on metabolic ward (weeks).
[b] β-Sitosterol recovery corrected for fecal flow variations by use of Cr_2O_3 data.
[c] Raw data obtained by Method IV (1), uncorrected.
[d] Raw data obtained by Method IV, corrected by two internal standards (17, 18).
[e] 100 − percent dose recovered in feces (corrected by two internal standards).
[f] Percent absorption corrected for re-excretion of absorbed labeled cholesterol. In Patients 1–4 it was not technically feasible to make this correction (see text: Methods, calculations).
[g] $^{14}C/^{3}H \times 100$, after normalization of doses, where n = 5–8 observations.
[h] (Area under ^{14}C-curve ÷ area under ^{3}H-curve) × 100, after normalization of doses.
[i] Sum of [percent dose recovered in feces (corr.) (fecal method)] plus [percent absorption by isotope ratio method].
[j] Patient 11 was studied under metabolic ward conditions for 8 months, and comparative absorption measurements were performed on two occasions, the first during a control period and the second during the oral administration of 150 mg of deoxycholic acid daily. Over this entire period, the recovery of Cr_2O_3 was precisely 100%. However, from day 54–61 and from day 127–134, when the Method IV studies were carried out, the recoveries of Cr_2O_3 and β-sitosterol were greater than 100%, due to fecal flow variations. When the actual recovery of β-sitosterol was corrected according to the Cr_2O_3 data, β-sitosterol recovery became 100%.

Method IV ("double-corrected" for fecal flow variations and neutral sterol degradation) was 36.6% (range 13.8–58.8%). However, in 10 experiments, it also was possible to calculate the re-excretion of absorbed labeled cholesterol administered orally. (In the remaining four patients this calculation was not possible, since they had been given labeled β-sitosterol orally.) In Table 4 the percent absorption data corrected for this re-excretion are listed as "triple-corrected". The net effect of applying this third correction factor was less than 2.5 percentage points in all cases, except in Patient 7 where the difference was 6 percentage points and where the triple-corrected figure agreed more closely with the isotope ratio figure than did the double-corrected one.

(*c*) *Isotope ratio data.* Mean percent absorptions by the isotope ratio method in the 12 technically acceptable studies was 42.1% (range 15.7–62.9%). The standard deviations around each of the means were small (±0.1–2.5), and the coefficients of variation averaged only 2.5% (0.2–3.9%). This indicates that the isotope ratios from day to day (after oral cholesterol curves had reached their peaks) were highly reproducible, and that subsequently the two decay curves were strictly parallel in every study.

Percent absorption by the plasma radioactivity method also was calculated by area integration of the two curves; these results are included next in Table 4. It is evident that the percent absorptions calculated in these two ways were almost identical in every case, which indicates that in man no significant error was introduced in the isotope ratio calculation by the somewhat slower appearance in the plasma of the orally administered radioactive cholesterol (identical results

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were seen in two cases, lower results by area integration in eight cases, and higher in two). The correspondences in these two sets of calculations were very similar to those reported in rats by Zilversmit and Hughes (6).

(*d*) *Precision check.* Total recoveries of orally administered labeled cholesterol were calculated (Table 4) as a composite check on the precision of the methods being compared. This was calculated by summing the unabsorbed labeled cholesterol determined by fecal analysis and the absorbed labeled cholesterol calculated by the isotope ratio method. In the 12 technically satisfactory studies, the mean recovery of administered radioactivity was 105.6% (range 94.4–125.3%), whereas in the three studies in which chromic oxide recoveries were less than 80%, the total recovery of labeled cholesterol averaged only 87.3%. The widest discrepancy (125.3% recovery) was seen in Patient 8 (see below).

(*e*) *Comparisons of two sets of absorption data.* When the absorption results obtained by the fecal radioactivity and plasma isotope ratio methods are compared (the two tests having been carried out simultaneously in all patients), it is found that individual comparisons were within 5 percentage points in 8 of these 12 experiments, and within 10 points in 11 experiments. Only in Patient 8 were the comparative results widely discrepant (22 percentage points) and in this patient the percent recovery of orally administered labeled cholesterol was also unacceptable (125%, see section *d* above), raising the question of a technical fault in this patient's study; however, it may also be noted that the duration of hospital admission of this patient was only 2.5 weeks, which raises the question whether a metabolic steady state had been obtained at the time the tests were carried out.

All comparisons are shown graphically in **Fig. 3,** in which the "double-corrected" Method IV data are plotted. The effect of making the "triple-correction" of Method IV data would be to bring the solid circles slightly closer to the line of identity. Nevertheless, the tendency to obtain higher absorption data by the isotope ratio method is evident: 8 of 12 comparisons fell to the left of the line of identity.

Statistical analysis of these 12 comparisons, made by estimating confidence intervals (23), shows that we could be 95% confident that the two absorption methods produced results within 5 percentage points, and 99% confident that the differences were less than 7 percentage points.

(*f*) *Cholesterol absorption in mg/day.* When cholesterol absorption was calculated in terms of mass (mg/day) and related to cholesterol intake in the 12 technically acceptable studies here reported, the plot of the data

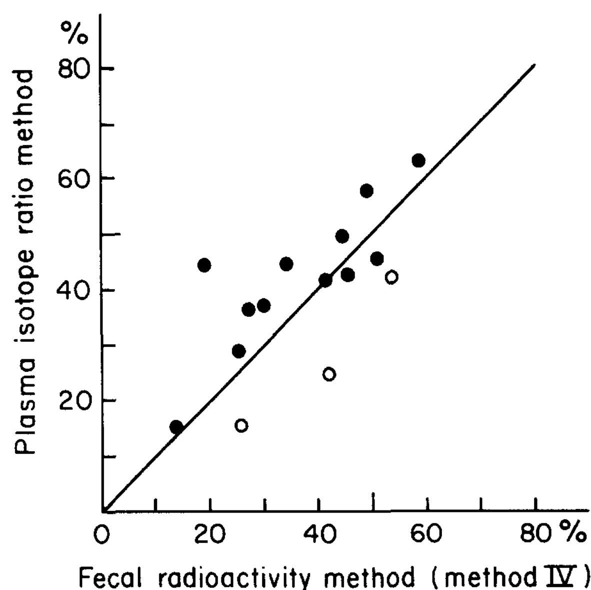

Fig. 3. Percent absorption of cholesterol by two methods in 15 studies plotted against the line of identity. Solid circles, technically satisfactory studies; open circles, unsatisfactory studies (see text). The Method IV data shown are those referred to in Table 4 as "double-corrected". For the technically acceptable studies, r = 0.83.

appeared to be linear over the small range of cholesterol intakes employed (75–721 mg/day) with r = 0.90. Previously reported studies by fecal radioactivity Methods I, II, and IV in 15 patients (24) showed the same linear relationship over an intake range of 30–3000 mg/day.

(*g*) *Test of the modified plasma method.* The earlier finding that the isotope ratios in plasma became parallel after 2–3 days prompted us to validate a shortened procedure in which only one blood sample was drawn after isotope administration, i.e., with omission of curve- and area-analysis. In six patients the mean percent absorptions obtained by the two approaches differed by less than 0.9 percentage points; this was well within 2 SD of the means obtained by analysis of the full curve.

DISCUSSION

Any attempt to quantify the key parameters of cholesterol homeostasis in a complex organism such as man must begin with a precise measurement of the amount of cholesterol that is absorbed by the intestinal tract. Since the absorption of this important compound is neither rapid (as in the case of the small water-soluble nutrients) nor complete (as in the case of dietary fats), there are a number of uncertainties that must be accepted by the investigator who seeks to

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quantify the process with any useful degree of accuracy. First, the mechanisms by which cholesterol is absorbed by the intestinal mucosa are still not clear, despite the enlightened efforts of many outstanding laboratories over the last three decades.

Second, the cholesterol to be absorbed has at least two major sources, both of which vary in amount from day to day, namely, the diet and the bile; mucosal cholesterol may also contribute. To this day it is not clear whether dietary cholesterol and biliary cholesterol are absorbed at the same rate.

Third, since the extent of cholesterol absorption is never complete, it is reasonable to suspect that the milieu of the succus entericus governs the quantitative aspects of absorption to a considerable degree. The composition of the succus is constantly changing through the day in relationship to meals and to secretions of different materials at successive levels of the gut, and the speed of its movement through the intestinal canal is not only variable but discontinuous.

Fourth, the size of the mucosal pool of cholesterol has never been measured in man in vivo, yet the passage of newly absorbed cholesterol through this pool must be affected not only by its size but also by the extent of arterial circulation through the mucosa and by the rate of formation and removal of chyle from the mucosa.

These uncertainties notwithstanding, it is generally agreed that the absorption of cholesterol is limited to its unesterified form, that the major site of absorption is the upper jejunum, and that the extent of absorption is negligible in the absence of bile. The peak of absorption into intestinal chyle occurs several hours later than that of dietary triglyceride; all absorbed cholesterol passes to the liver as cholesteryl esters in chylomicrons via the lymphatics (except in birds and reptiles, in which the thoracic duct system is vestigial); and chylomicron cholesterol is removed in the liver and leaves it either as very low or low density lipoprotein moving into the plasma, or as free cholesterol moving into the bile canaliculi.

In the setting of these many vagaries and some facts, we may now examine the objective this laboratory has set for itself, and ask how best to measure the habitual day-to-day extent of cholesterol absorption in man after some reasonable constancy of dietary intake has been assured and physiological variations have been minimized, that is, during some sort of steady state lasting not days but weeks. In this steady state we assume that all other rate processes involved in cholesterol regulation are also relatively unvarying. We are not the first to conclude that the most reliable approach would appear to be a measurement of the difference between the intake of cholesterol and the recovery in feces of unabsorbed dietary cholesterol, where the distinction between exogenous and endogenous cholesterol is accomplished by use of tracers labeling one or the other of these sources. However, we were able to prove that the accuracy of such measurements depended critically on taking account of the extent of degradation of neutral sterols to nonsterols during their transit through the intestinal tract (17). Consequently, we developed the means to measure the extent of this degradation, so as to multiply the mass of fecally excreted nonabsorbed dietary cholesterol by a factor representing those degradative losses.

These conditions have been met in several ways that have been compared in detail in a previous publication (1). Now, six years later and in the light of the present new experiments with the plasma isotope ratio method, it seems appropriate to re-assess the various methods, their advantages and shortcomings. In Method I the endogenous pools of cholesterol are labeled by previous intravenous administration of radioactive cholesterol. Then, by comparing the excreted fecal radioactivity to the specific activity of plasma cholesterol, the mass of labeled cholesterol excreted daily can be deducted from total daily neutral steroid output. This results in a figure (mg/day) for unabsorbed (unlabeled) dietary cholesterol. It is a basic assumption of this approach that biliary (labeled) and exogenous (unlabeled) cholesterol are equally well absorbed. Since the measurement process can be repeated at daily intervals as long as measurable amounts of radioactivity remain in the plasma and in the feces, the investigator achieves a figure for cholesterol absorption that is the mean of many determinations, but he also obtains a coefficient of variation around this mean that represents the sum of methodological *and* physiological variability.

Method I has the advantage of simplicity, but there are drawbacks that we have previously described in detail (1). After intravenous injection of radioactive cholesterol, calculations of absorption can be made almost immediately and repeatedly thereafter, but their reliability will be greatest after the specific activity–time curve becomes log-linear, and this usually takes 6–8 weeks. It is a major disadvantage of Method I that the transit time of intestinal contents must either be measured or (less desirable) simply assumed, for the calculation depends on knowing the plasma cholesterol specific activity at the exact time that labeled biliary cholesterol is secreted into the intestinal lumen. When the specific activity–time curve is falling rapidly, or if the turnover of intestinal contents is sluggish (1), or both, results by Method I will have doubtful validity.

Method II employs continuous oral labeling with ra-

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dioactive cholesterol in order to quantify the mass of unabsorbed exogenous cholesterol. Measurements are made of the specific activities of plasma cholesterol, of total fecal neutral steroids, and of the mass of total fecal neutral steroids; the mass of unabsorbed cholesterol is calculated by simultaneous equations (1). (As with Method I, it is assumed that endogenous and exogenous cholesterols are equally well absorbed.) The method may be applied at any time after 4 days of feeding radioactive cholesterol; the isotope steady state need not be attained. We have previously discussed the evidence that leads us to the conclusion that Method II is superior to Methods I, III, and IV in precision and reproducibility (1). The main advantage is that the unabsorbed dietary cholesterol is calculated directly and with great precision because the major proportion of excreted radioactivity is in that fraction; furthermore, no correction need be made for undefined lags in transit of intestinal contents. However, the feeding of a constant daily dose of radioactive cholesterol for a minimum of three or four stool collection periods demands metabolic ward conditions and a minimum time of 2 weeks for each patient tested. Time in hospital may be minimized by starting stool collections with the first appearance of a carmine marker given with the first formula feeding.

Method III is similar in practice to II, except that it must be carried out long enough to attain the isotopic steady state, at which time the calculation of absorption becomes theoretically sound. We have abandoned this approach in our clinical studies because the time required to reach the isotopic steady state in man is very long (100 or more days).

Method IV and the plasma isotope ratio method have been described in detail in the present report. Neither method requires the assumption that exogenous and endogenous cholesterols are equally well absorbed. Method IV has the advantage over Methods I and II of simplicity and speed; nevertheless, metabolic ward conditions are required in order to assure a constant measured cholesterol intake and to make complete stool collections for 6–8 days. Still further improvements are offered by the plasma isotope ratio method: avoidance of metabolic ward conditions and of fecal analyses, and minimal inconvenience for patients. However, both methods yield only one figure for absorption, with no impression gained of physiological variability during the testing period through the statistics of a coefficient of variation. In other words, the result obtained in both cases pertains solely to the fate of the single bolus of labeled cholesterol administered orally on day 1, and the results obtained are truly representative of daily absorption before, during, and after the test, *only* if the patient is in the metabolic steady state. Both procedures lend themselves to repetition as early as 3–4 weeks, an advantage not shared by Methods I and II.

Over the past 10 years we have carried out a total of 15 comparisons of Methods I, II, and IV, the results of which have been published piecemeal in three other reports (1, 25, 26). Method I was compared to IV in eight patients and the mean difference was 19.9% (1.8–37.8%). Method II was compared to IV in four patients and the mean difference was 13.2% (1.1–26.7%). Method I was compared to II in three patients, with a mean difference of 15.0% (8.1–18.9%). A more meaningful evaluation of the precision of Methods I and II is obtained by comparing the coefficients of variation that these techniques afford. In 46 absorption studies carried out by Method I and described piecemeal in seven other papers (1, 11, 17, 24–27), the mean coefficient of variation was 30.4%. However, in 21 studies carried out by Method II that were gathered out of five separate papers (1, 11, 24, 25, 27), the mean coefficient of variation was only 21.4%. These data confirm our earlier conclusion that Method II affords the greatest accuracy and precision, and that results obtained by Method IV compare favorably with those obtained by Method II. Finally, the present report signifies that the plasma isotope ratio method gives results that compare closely to those obtained by Method IV.

Thus, we may conclude that (*a*) for all of the available absorption procedures, an accurate index of daily cholesterol intake is required in order to calculate absorption in absolute terms (mg absorbed/day); and (*b*) percentage absorption data obtained in man in the metabolic steady state by Methods I, II, IV, and the plasma isotope ratio method are essentially similar.[3] (*c*) The fact that Method I results resemble so closely those of Methods II and IV furnishes us with indirect evidence that endogenous and exogenous cholesterols are absorbed at similar rates, since Method I measures the absorption of endogenous

[3] However, it is a matter of some concern that falsely high results for absorption would be obtained if simple isotope exchange occurred between labeled luminal cholesterol and unlabeled mucosal cholesterol. Grundy and Mok (28) have addressed this concern directly; they infused cholesterol-containing test meals into the upper duodenum, collected samples at two loci in the upper jejunum, and calculated net absorption of cholesterol as well as isotope exchange per unit time. In 10 patients they found that the specific activity of the luminal cholesterol was lower at the second sampling site than at the first. This could have been due either to isotope exchange or to secretion into the lumen of unlabeled mucosal cholesterol, with the first possibility considered to be much more likely than the second. Now, if isotope exchange does take place routinely, Methods I, II, IV, and the plasma isotope ratio method would all yield spuriously high results. It remains for future studies to examine the extent of this error in finer detail.

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cholesterol, while II, IV, and the isotope ratio method all measure the absorption of exogenous cholesterol. (*d*) Further, we conclude that physiological variations in absorption during the test period are measurable only by Methods I and II.

Yet, it is in the setting of physiological or induced variability that we are now most eager to be able to measure cholesterol absorption reliably, that is, when cholesterol intakes are varied, during drug administration, with fat and carbohydrate exchanges, and in the face of variations in bowel physiology. For these and other reasons, we feel it is now necessary to explore in man the limitations of the isotope ratio method, since the usefulness of the method will be determined by its applicability in disease states, in conditions of hyperlipidemia, and in the context of defining the mode of action of cholesterol-lowering regimens.

The reproducibility of results obtained by the isotope ratio method needs to be defined in patients maintained in the metabolic steady state. The effects of intentional perturbations of that steady state on day one of the test should be explored, as should the effects of administering both the oral and intravenous test doses in other vehicles and at various times of day in relation to meals. The optimal sampling time for plasma isotope ratio measurements should be sought, whether fasting or postprandial. What is the validity of the procedure during altered states of cholesterol synthesis, i.e., during fasting and with cholestyramine dosage? Is the technique valid in patients with upper intestinal bacterial overgrowth, when partial degradation of the oral dose of labeled cholesterol may occur prior to its absorption? These questions are currently being tested in our laboratory.

The data we have reported represent an encouraging beginning in exploring the potential usefulness of this new method for measuring cholesterol absorption in man. The advantages of the technique and the compelling need for precision in quantifying this critical determinant in cholesterol homeostasis stimulate us (and we hope other investigators) to explore the problems that remain.

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