An evaluation of four methods for measuring cholesterol absorption by the intestine in man

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ABSTRACT Critical comparisons have been made in 12 patients of four methods for measuring cholesterol absorption from the intestine. Methods I–III depend on the use of labeled cholesterol (intravenously or continuous labeling orally) in conjunction with sterol balance measurements; Method IV can be carried out with only a single test dose containing labeled cholesterol plus labeled β-sitosterol. In the latter technique absorption is calculated as the loss of cholesterol relative to β-sitosterol during intestinal transit.

Method III (isotopic steady-state method) proved to be undependable because of uncertainties in determining the existence of an isotopic steady state. However, Method IV gave good agreement with Methods I and II, and it appears to have certain practical as well as theoretical advantages. Although Method IV requires collections of stools for up to 8 days, it is nevertheless the most rapid and the simplest of all the methods for estimating absorption. It can also be used in certain situations, such as in fur-licking animals, when Methods I and II are inadequate. Therefore, this method would seem to be a valuable addition to other isotopic techniques for estimating cholesterol absorption in man.

SUPPLEMENTARY KEY WORDS cholesterol-4-^{14}C · β-sitosterol-22,23-^{3}H · isotopic steady state · sterol balance method · isotope kinetics · fur-licking animals

THE FEEDING of cholesterol to animals of certain species such as rabbits and fowl produces a marked hypercholesterolemia; in other species, including man, the response of plasma cholesterol to dietary cholesterol is much less striking. Although it is now generally accepted that the addition of appreciable quantities of cholesterol to the diet of human beings usually causes significant increases in levels of plasma cholesterol, the magnitude of these changes rarely exceeds 20% (1–4) and is often much less; these increments are small when contrasted to increases of several hundred percent in other species. Therefore, in man, certain mechanisms must have evolved that compensate for large loads of dietary cholesterol so as to prevent the development of high-grade hypercholesterolemia. Various mechanisms can be imagined that might prevent a rise in plasma cholesterol: (*a*) limited absorption of dietary cholesterol; (*b*) increased reexcretion of absorbed cholesterol in the form of neutral or acidic steroids; (*c*) decreased synthesis of cholesterol; and (*d*) some restriction on the transport capacity of circulating lipoproteins that has the effect of keeping plasma levels low in the face of increasing body pools.

Efforts have been made in recent years in several laboratories to examine the importance of such mechanisms for the prevention of hypercholesterolemia. Logically, the first problem for investigation is that of absorption, because, if absorption of cholesterol in man is extremely limited, other possible protective mechanisms may fade into insignificance.

In the few studies in man reported to date, the various techniques employed to measure absorption under diverse experimental conditions have given rise to equally various results and interpretations. For example, Kaplan, Cox, and Taylor (5) and Wilson and Lindsey (6) used isotope steady-state methods and estimated that maximum daily absorption of dietary cholesterol did not exceed 450 mg/day.[1] On the other hand, Borgström (7), who fed a single dose of radioactive cholesterol and con-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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[1] The upper limits noted by Wilson and Lindsey (6) in their text were 320 and 340 mg/day for their two normal young men. However, our recalculation of the data in their Table 1 indicated 456 and 304 mg/day, respectively, for the two men on high-cholesterol intakes.

sidered absorption equal to the radioactivity not recovered in the feces, concluded that absorption was directly related to the dose of cholesterol fed and that, within experimental limits, there was no ceiling on cholesterol absorption. We reported a detailed study in one patient who was fed large amounts of cholesterol in the diet for a long period; absorption measurements were carried out by a combination of chromatographic and isotope balance methods (8). Early in the period of cholesterol feeding, values for absorption were consistently greater than 500 mg/day, but at later times absorption averaged less than 400 mg/day. Although our more recent experiments (9) suggested to us that in man there is a rather low ceiling for absorption of exogenous cholesterol, we would stress that the limits had not been well enough defined.

Therefore, as a prelude to further studies on the interaction of absorption, synthesis, and excretion of cholesterol in man, we have attempted to reevaluate the various methods that have been introduced for the measurement of cholesterol absorption. In a previous study (9) we examined in detail two techniques (Methods I and II) for estimating cholesterol absorption; these are based on the combined use of sterol balance and isotopic balance methods. In the present paper we have evaluated two more absorption methods: (*a*) the isotopic steady-state technique (Method III) described by Wilson and Lindsey (6), and (*b*) the use of a single oral dose of radioactive cholesterol (Method IV) introduced by Borgström (7). These latter two methods have been compared to Methods I and II, and the advantages and disadvantages of each are described.

METHODS

Patients

Studies were carried out on 12 patients hospitalized on a metabolic ward at The Rockefeller University Hospital. The age, sex, body build, caloric intake required for energy balance, and clinical diagnosis are listed in Table 1. Of nine patients, with one or another form of hyperlipoproteinemia, on solid food diets six had evidence of ischemic heart disease. There was, in addition, one patient with primary biliary cirrhosis and two with abetalipoproteinemia. Patients were hospitalized for periods of 10–125 days.

Diets

In all but one case (patient 7) the food intake consisted exclusively of orally administered liquid formula feedings, a regimen described elsewhere (12, 13). All formulas provided 15% of total calories as protein, 5, 20, or 40% as fat, and the remainder as glucose. Patient 6 was

TABLE 1 CLINICAL DATA

Patient	Initials	Age	Sex	Height	Weight		Calories	Diagnosis
		yr		*cm*	*kg*	*% of ideal**		
1	N.H.	34	M	174	74.0	94	2350†	Endogenous hyperglyceridemia (type V),‡ asymptomatic
2	M.R.	60	M	167	67.0	88	2380	Endogenous hyperglyceridemia (type V), IHD§
3	J.J.	42	F	161	72.0	114	2700	Endogenous hyperglyceridemia (type V), asymptomatic
4	D.R.	56	F	157	54.0	87	1930	Endogenous hyperglyceridemia (type V), asymptomatic
5	L.S.	51	F	159	51.0	81	2800	Primary biliary cirrhosis
6	P.B.	5 mo	M	65	5.3	65	1100	Abetalipoproteinemia
7	R.B.	10	M	125	29	112	—	Abetalipoproteinemia
8	R.W.	54	F	155	79.5	132	2200	Hypercholesterolemia, IHD
9	Y.R.	55	F	146	58.9	107	1800	Hypercholesterolemia and endogenous hyperglyceridemia, xanthomatosis, IHD
10	E.D.	59	F	145	43.4	77	1600	Hypercholesterolemia, xanthomatosis, IHD
11	A.R.	56	F	163	60.8	92	2150	Hypercholesterolemia and endogenous hyperglyceridemia, xanthomatosis, IHD
12	F.L.	67	F	156	65.0	105	2160	Hypercholesterolemia, xanthomatosis, IHD

* According to life insurance tables (10).
† Caloric intake required to maintain constant body weight.
‡ Typing of hyperlipoproteinemias according to Fredrickson, Levy, and Lees (11).
§ IHD, ischemic heart disease.

fed a fat-free formula supplemented with one egg yolk each day. Patient 7 ate a low-fat diet of solid foods; intakes were not measured.

Table 2 lists the different dietary fats used in formula diets and the sterol intakes on those diets. Higher intakes of cholesterol were achieved by dissolving crystalline cholesterol (Mann Research Laboratories, New York) in the oil phase of diet A before the bulk preparation of this formula.

Radioactive Sterols

Cholesterol-1,2-^{3}H and cholesterol-4-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. β-Sitosterol-22,23-^{3}H was prepared by Dr. M. J. Thompson, Beltsville, Md., and Dr. G. Gupta of The Rockefeller University. β-Sitosterol-4-^{14}C was obtained from Amersham/Searle Corp., Des Plaines, Ill. All four radiosterols were subjected to TLC on Florisil with ethyl ether–heptane 45:55 (v/v); less than 4% of radioactivity remained at the origin in all cases, and only the material that chromatographed with the same R_F as a pure sterol standard was administered to patients.

Analytical Methods

Plasma cholesterol was measured by the method of Block, Jarrett, and Levine (14) on the AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N.Y.). Specific activity of plasma cholesterol was determined by counting a portion of the same extract used for mass measurement in a Packard Tri-Carb scintillation counter (model 3003) as previously described (15).

Fecal steroids were measured by methods developed in this laboratory (15, 16); values obtained for excretion of neutral steroids were corrected for losses during intestinal transit and for variations in fecal flow rates with unlabeled dietary β-sitosterol as an internal standard (17, 18). Excretions of acidic steroids were corrected for variations in fecal flow by the use of constant daily doses of chromic oxide as an internal standard, as described earlier (19). In patients 8 and 12, recoveries of chromic oxide were below 90% (Table 3); they would not be considered to be ideal for balance studies according to the criteria of Davignon, Simmonds, and Ahrens (19). Nevertheless, we justify the inclusion of these patients here on the fact that the recovery of unlabeled β-sitosterol was similar to that of chromic oxide in every pool throughout the entire collection period. Thus, we believe that any losses of neutral and acidic sterols were due to incomplete fecal collections or to incomplete colonic emptying.

Tissue Sterols. Specific activities of tissue cholesterol were measured by a modification of the method used for fecal neutral steroids (16). Approximately 1 g of tissue (muscle, skin, adipose tissue, or xanthoma) was refluxed for 1 hr in a 125-ml glass-stoppered bottle containing 20 ml of N NaOH in 90% ethanol. After refluxing, 10 ml of water was added, and the cholesterol was extracted with three 50-ml portions of petroleum ether (bp 60–80°C). After evaporation of the solvent, the samples were transferred to Florisil thin-layer plates and developed in ethyl ether–hexane 55:45. The band corresponding to cholesterol was identified with rhodamine and eluted with ethyl ether. After evaporation of the ethyl ether, 5α-cholestane was added as an internal standard for GLC; one aliquot was assayed for radioactivity and another for mass by GLC of the trimethylsilylether (16).

TABLE 2 DIETARY FATS AND STEROL CONTENTS OF THE FORMULA DIETS

Diet	Dietary Fat and % of Total Caloric Intake	Sterol Content of Diet			Source of Plant Sterols
		Cholesterol	Total Plant Sterols	β-Sitosterol	
		mg/1000 cal			
A	Cottonseed oil, 40	12	160	142	Inherent
B	Lard oil, 40	237	221	144	Added*
C	Corn oil, 20	12	200	130	Inherent
D	Low-fat,† 5	130	90	60	Added*

* β-Sitosterol (Mann Research Laboratories, New York), purified and prepared in microcrystalline form by Dr. Erold R. Diller, Eli Lilly Co., Indianapolis, Ind.; the final product contained 65% β-sitosterol, 30% campesterol, and 5% stigmasterol.

† One egg yolk and 100 mg of plant sterols were added to a fat-free formula each day.

TABLE 3 PERCENTAGE RECOVERY OF INTERNAL STANDARDS IN EACH PATIENT

Patient	β-Sitosterol	Chromic Oxide	Loss of β-Sitosterol*
1	84†	100	16
2	72†	—	—
3	97†	—	—
4	78†	—	—
5	102†	—	—
6‡	58§	—	—
7	103§	—	—
8	70§	78	10
9	85§	111	18
10	93§	91	0
11	76§	93	19
12	70,§ 66§	59, 69	5‖

* Percentage loss of unlabeled (or radioactive) β-sitosterol relative to the recovery of chromic oxide (19).

† Recovery of β-sitosterol-^{3}H in an 8-day collection of feces after administration as a single dose.

‡ In this infant the collection of feces was known to be incomplete.

§ Recovery of unlabeled dietary β-sitosterol as percentage of constant daily intake in formula diets.

‖ Mean loss over both experimental periods.

Measurement of Cholesterol Absorption

Absorption by Methods I, II, and IV is based on measuring the difference between dietary intake and unabsorbed dietary neutral steroids in feces. The three methods differ according to the way in which the amount of unabsorbed dietary sterol is calculated.

Method I. By this method, values for absorbed dietary cholesterol are obtained as follows:

Exogenous cholesterol absorbed daily (mg/day) = daily cholesterol intake (mg/day) − daily unabsorbed dietary cholesterol (mg/day) (Eq. 11, Ref. 9), where daily unabsorbed dietary cholesterol (mg/day) = daily fecal total neutral steroids (mg/day) − daily fecal endogenous neutral steriods (mg/day) (Eq. 10, Ref. 9). Daily fecal total neutral steroids are determined by combined thin-layer and GLC as described above (16); after administration of a single intravenous dose of radioactive cholesterol, daily fecal endogenous neutral steroids are estimated isotopically by dividing the total radioactivity in the fecal neutral steroid fraction (dpm/day) by the specific activity of plasma cholesterol (dpm/mg) 1–2 days previously (Eq. 4, Ref. 9).

The intravenous dose of radioactive cholesterol was administered at the beginning of each patient's study; 1 ml of ethanol containing a known amount of radioactive tracer (approximately 100 μCi) was dispersed in 150 ml of physiologic saline, and the mixture was immediately administered intravenously. The flask and tubing were assayed for residual radioactivity, and the dose administered was appropriately corrected. Specific activities of plasma cholesterol were determined twice weekly thereafter.

Method II. This procedure employs continuous oral labeling with radioactive cholesterol. The cholesterol absorbed daily is calculated as the difference between cholesterol intake and unabsorbed dietary cholesterol, and the latter is determined by simultaneous equations as follows: $Z = X + Y$, where Z = total fecal neutral steroids (mg/day), X = fecal neutral steroids of endogenous origin (mg/day), and Y = unabsorbed dietary cholesterol (mg/day) (Eq. 15, Ref. 9); and $Z \cdot SA_{(z)} = X \cdot SA_{(x)} = Y \cdot SA_{(y)}$, where $SA_{(z)}$ = specific activity of total fecal neutral steroids, $SA_{(x)}$ = specific activity of plasma cholesterol, and $SA_{(y)}$ = specific activity of dietary cholesterol (Eq. 16, Ref. 9). The equations are solved for Y. This method may be used at any time after 4 days of feeding radioactive cholesterol; the isotope steady state need not be attained.

Radioactive cholesterol used in oral labeling was incorporated into the formula diets in order to assure a continuous intake of radiosterol for many weeks with great precision. 10 ml of ethanol containing the tracer was added to 40-kg batches of each formula during its homogenization. Thus, the patient received exactly the same amount of isotopic cholesterol every day, divided into five equal portions. Aliquots of all formulas were tested for homogeneity in terms of sterol content and specific activity.

Method III. By this method, which also makes use of constant oral labeling with radioactive cholesterol, it is theoretically required that the isotopic steady state be attained (6). Absorption can be estimated only during the isotopic steady state. Daily absorption of dietary cholesterol (mg/day) = daily cholesterol turnover (mg/day) × fraction of plasma cholesterol derived from absorbed dietary cholesterol (Eq. 18, Ref. 9). The daily cholesterol turnover can be determined either by sterol balance methods (Eq. 7, Ref. 9) or from specific activity–time curves of plasma cholesterol using the two-pool model (Eq. 3, Ref. 9); in the present study sterol balance methods were used. The fraction of plasma cholesterol derived from absorbed dietary cholesterol is represented by the quotient of specific activities of plasma cholesterol and dietary cholesterol in the isotopic steady state.

Method IV. With this method the absorption of dietary cholesterol was calculated following oral administration of a single dose of radioactive cholesterol and was calculated as the percentage of the dose not recovered in feces. However, the percentage absorption was calculated only after losses from sources other than absorption were corrected for, using orally administered radioactive β-sitosterol as an internal standard. The following equation was used: Percentage of dietary cholesterol absorbed = [1 − (radioactivity in fecal cholesterol ÷ radioactivity in fecal β-sitosterol) × (radioactivity in administered β-sitosterol ÷ radioactivity in administered cholesterol)] × 100.

A mixture of radioactive sterols was dissolved in 1 ml of ethanol and added to the 2:00 PM formula feeding with magnetic stirring. This was the third feeding of the day; the other four feedings on the test day were unlabeled. Each test dose consisted of about 1 μCi of each radiosterol. After its administration all stools were collected for 8 days and pooled; the radioactivities in neutral steroids were measured on an aliquot of this pool.

For many studies, three labeled compounds were used simultaneously: radioactive cholesterol for Methods I, II, and III, and labeled cholesterol plus labeled β-sitosterol for Method IV. Because the two sterols labeled with ^{14}C or ^{3}H were available to us, we used cholesterol labeled with one isotope for Methods I, II, and III, and for Method IV both cholesterol and β-sitosterol were labeled with the other isotope. Then, in order to measure the relative proportions in feces of cholesterol and β-sitosterol (or their conversion products, the 5β-H compounds) labeled with the same isotope, it was necessary to isolate the two radiosterols quantitatively; this was

done after preliminary isolation of the major fecal neutral steroid band by TLC on Florisil with ethyl ether–heptane 45:55, with subsequent separation of cholesterol from β-sitosterol by reversed-phase TLC. For this we modified the method of de Souza and Nes (20) by running the chromatograms at 4°C and then visualizing the sterol bands with rhodamine spray; we eluted the separated sterols from the plate with ethyl ether. In our hands, the two sterols were separated with less than 4% cross-contamination.

Table 4 summarizes certain experimental variables in our studies of cholesterol absorption by four different methods in 12 patients; included are the duration of study, diet fed, cholesterol intake, and isotope administered in each experiment. Daily absorption of dietary cholesterol was measured continuously in patient 12 by Method I and in patients 8–11 by Method II. Estimations were also made by Method III in the latter group of patients while they were receiving constant intakes of radioactive cholesterol by mouth. Multiple determinations of cholesterol absorption were carried out by Method IV in patients 8–12; single tests by Method IV were performed in patients 1–7.

RESULTS

1. Pattern of Fecal Excretion of Radioactive Sterols

Table 5 shows the patterns of excretion in feces of cholesterol-^{14}C and β-sitosterol-^{3}H in patients 1–7 following simultaneous oral administration of a single dose of each sterol. In these pilot experiments all stools were collected separately for 5–7 days after administration of the labeled sterols; some patients failed to have stools daily, and patients 2, 6, and 7 had no stools for the first 2 or 3 days of the study. Table 5 presents the percentage of the total administered dose of each isotopic sterol that was excreted in successive stools, together with the ratios of the two radioactivities in each sample. Total recoveries of the two radioactive sterols are also shown, along with calculations of cholesterol absorption made in two ways: (*a*) from the data obtained on the first stool excreted following the administration of radioactive sterols, and (*b*) from the data pooled for the entire study. In both cases, absorption was calculated as the percentage change in the ratio of radioactivities in the two sterols, relative to that of the mixture fed.

Even though β-sitosterol is essentially nonabsorbable (21), it should be noted that in most cases the recoveries of β-sitosterol were incomplete. In previous studies (17, 18) we have demonstrated that in many patients appreciable amounts of cholesterol and β-sitosterol are lost in their passage through the intestine, presumably due to destruction of the steroid ring by intestinal microorganisms. Nevertheless, losses of the two sterols have been shown to be quantitatively identical and, therefore, any losses of cholesterol occurring within the intestinal tract (other than those due to absorption) can be corrected by using dietary β-sitosterol as an internal standard. In the present experiments the calculated values for cholesterol absorption would have been erroneously high, had the degradative losses of neutral steroids not been corrected according to the recovery in the feces of β-sitosterol.

Sequential analysis of fecal samples in patients 1–7

TABLE 4 EXPERIMENTAL VARIABLES IN A COMPARISON OF FOUR ABSORPTION METHODS IN 12 PATIENTS

Patient	Duration of Study	Diet	Daily Cholesterol Intake	Method I (single dose, intravenous)*	Methods II and III (daily oral labeling)†	Method IV (single oral dose)
	days		*mg/day*	*μCi*	*μCi/day*	*mixture*‡
1	21	B	543			3
2	125	B	550			3
3	10	C	32			3
4	12	C	24			3
5	49	B	667			3
6	90	D	143			3
7	10	low-fat solids	unknown			3
8	77	A	2936		0.94	1
9	86	A	2471		0.79	1
10	63	A	2119		0.68	1
11	66	A	532		1.60	1
12	50	A	241	108		2
	44		909			2

* Cholesterol-1,2-^{3}H.
† Cholesterol-4-^{14}C.
‡ Mixture 1, β-sitosterol-22,23-^{3}H plus cholesterol-1,2-^{3}H; mixture 2, β-sitosterol-4-^{14}C plus cholesterol-4-^{14}C; mixture 3, β-sitosterol-22,23-^{3}H plus cholesterol-4-^{14}C. About 1 μCi of each labeled sterol was given per test dose.

TABLE 5 NONSYNCHRONOUS TRANSIT OF CHOLESTEROL-^{14}C AND β-SITOSTEROL-^{3}H THROUGH INTESTINAL CANAL AFTER SIMULTANEOUS ORAL ADMINISTRATION

Patient	Isotopes, Isotope Ratios and Days	Excretion of Administered Dose in Successive Stools*							Total	Calculation of % Absorption Based on Excretion of:	
		%							%	*first stool*	*all stools pooled*
1A	^{14}C	30.2	29.1	3.1	5.3	0.02			67	27	18
	^{3}H	41.3	34.8	2.7	4.1	0.18			83		
	^{14}C/^{3}H	0.73	0.83	1.14	1.29	0.11					
	Days†	(1)	(3)	(4)	(4)	(6)					
1B	"	19.2	3.9	36.0	1.0	1.3	1.8		63	40	24
		32.4	5.3	44.7	0.7	0.6	0.4		84		
		0.59	0.73	0.80	1.42	2.16	4.50				
		(1)	(2)	(4)	(5)	(6)	(7)				
2	"	57.2	3.9						61	20	15
		71.3	0.8						72		
		0.80	4.87								
		(3)	(5)								
3	"	14.3	22.0	11.0	2.8	0.5	2.0	0.48	53	56	45
		32.7	43.9	16.8	2.2	0.2	0.9	0.16	97		
		0.44	0.50	0.65	1.27	2.50	2.22	3.0			
		(1)	(2)	(3)	(3)	(4)	(5)	(6)			
4	"	33.9	5.8	6.6	0.6	0.4			47	44	39
		61.4	9.3	6.7	0.2	0.2			78		
		0.55	0.62	0.98	3.00	2.00					
		(1)	(1)	(2)	(4)	(4)					
5	"	18.0	70.2	5.2	6.2	1.6	0.0		101	4	1
		17.9	74.5	5.5	3.4	1.4	0.0		102		
		1.00	0.94	0.94	1.82	1.14	0.0				
		(1)	(2)	(3)	(4)	(5)	(6)				
6	"	33.1	4.5	2.0	5.6	6.0	3.0	0.1	54	36	7
		51.6	2.9	0.8	1.5	0.9	0.3		58		
		0.64	1.55	2.50	3.73	6.66	10.00				
		(2)	(3)	(4)	(5)	(6)	(7)	(8)			
7	"	45.5	19.8	18.1					83	47	19
		86.6	11.8	4.8					103		
		0.52	1.67	3.77							
		(3)	(6)	(8)							

* Percentage of administered dose excreted with each successive passage of feces (all stools were collected individually).
† Number in parentheses = number of days after oral administration of radiosterol mixture.

indicated that the ratios of ^{14}C/^{3}H were lowest in the first stool, and thereafter the ratios increased with each successive passage of feces. We believe that the low ratio of ^{14}C/^{3}H found in the first stool reflects the disappearance of labeled cholesterol into the intestinal mucosa by isotope exchange, and that the rising ratio thereafter indicates the back-exchange of some of this labeled cholesterol into the intestinal lumen from the mucosa. This explanation is strengthened by the findings in two patients with abetalipoproteinemia (patients 6 and 7), in whom absorption of cholesterol should be greatly reduced because of a defect in formation of chylomicrons; indeed, at no time could we detect isotopic sterol in the plasma. Nevertheless, there was definite asynchrony in the fecal excretion of the two radioactive sterols, suggesting that isotopic cholesterol was temporarily exchanged into the intestinal mucosa before its excretion into feces. A still different pattern of excretion was noted in patient 5, who suffered from obstructive liver disease (primary biliary cirrhosis). This patient failed to absorb any dietary cholesterol at all, and the unchanging ^{14}C/^{3}H ratios indicated the absence of iostope exchange with mucosal cholesterol (as well as of net uptake), as would be expected when the content of intraluminal bile salts fails to reach the critical micellar concentration.

In patients 1–4 the low isotope ratios in the first samples of feces (due to isotope exchange) resulted in values for cholesterol absorption that were significantly higher than those calculated for the 8-day pools. These were disappointing findings: if we had found the same isotope ratio in each specimen of feces collected after administration of the test mixture, we could have measured cholesterol absorption by Method IV on stools collected casually, rather than completely. However, the data in Table 5 demonstrate the necessity for making a prolonged and continuous collection of feces in order to calculate the true absorption of isotopic cholesterol; we chose an 8-day period for routine testing because in patients 1–7 there was no significant excretion of the radiosterols after 6 days. Furthermore, we concluded

TABLE 6 COMPARISON OF METHODS I, II, AND IV FOR MEASUREMENT OF CHOLESTEROL ABSORPTION

Patient		8	9	10	11	12A	12B
Duration of balance (days)		77	86	63	67	50	44
Days of fecal analysis: number of determinations		69:9	78:10	55:16	59:15	42:6	33:5
Cholesterol intake (mg/day)		2936	2387	2119	542	241	930
Cholesterol absorption (mg/day ± SD)							
Method I						113 ± 32	390 ± 57
Method II		724 ± 119	568 ± 147	744 ± 116	292 ± 41		
Method IV		732 ± 66	775 ± 155	722 ± 75	228 ± 31	111 ± 10	294 ± 47
		(4)*	(4)	(4)	(5)	(4)	(3)
Difference between Method I or II vs. Method IV	(mg)	8	207	22	66	2	96
	(%)	−1	−26	+3	+22	+2	+24

* Number in parentheses = number of separate determinations.

that an accurate comparison of Method IV with the other methods could be made only if we counted all the isotopic cholesterol passed through the intestinal tract into feces, whatever its temporary delay in the mucosa due to isotopic exchange. Indeed, in the employment of Methods I, II, and III any isotopic exchange that occurred would not be detected, since the calculations are made after isotopic equilibrium between mucosal, luminal, and plasma cholesterol is established.

Although we have attributed the rising $^{14}C/^{3}H$ ratios in successive fecal collections to back-exchange of labeled cholesterol from the mucosa, we also had to consider the possibility that some of the absorbed cholesterol-^{14}C was reexcreted into the intestinal contents through the biliary tract. While it is likely that recirculated radioactive cholesterol contaminated the 8-day collections of feces to some extent, the pattern of daily excretion shown in Table 5 suggests that this phenomenon contributed very little to the total excretion of radioactive cholesterol during this 8-day period. For example, in the studies of patients 1, 3, and 4 the fraction of administered radioactive cholesterol excreted in the feces in the final days was extremely small. Furthermore, in other patients on continuous stool collections the excretion of radioactivity after 8 days has been less than 1% of the administered dose, presumably due to the dilution of the absorbed test dose by large pools of exchangeable cholesterol within the body. Indeed, if significant amounts of absorbed labeled cholesterol were rapidly reexcreted in bile before equilibrating with body pools, the values for cholesterol absorption by Method IV would be systematically lower than those by all other methods; as will be shown below, this was not the case.

II. Comparison of Method IV with Methods I and II

Table 6 shows the values for cholesterol absorption obtained by Method IV compared with those by Methods I and II in patients 8–12; absolute and percentage differences are given. Fig. 1 shows the separate measurements made from each patient through the long periods each was studied (all data obtained by Method III will be considered below in Section III).

In patients 8, 10, and 12A the results obtained by Method IV were almost identical to those of Methods I or II. In patients 9, 11, and 12B the results obtained by all methods were variable, but in absolute terms the differences were rather small; the variations in absorption appeared to be independent of variations in total cholesterol intake.

Fig. 2 shows two situations in which systematic differences were obtained between Methods II and IV. This study in patient 8 included three dietary periods: in period I the diet was essentially free of cholesterol; in period II the diet contained 2936 mg/day of radioactive cholesterol; and in period III the patient returned to a cholesterol-free diet. Absorption of dietary cholesterol was measured by Method IV once during period I, four times in period II, and once in period III. In every test the patient was given a single dose of cholesterol-^{14}C (600 mg) and β-sitosterol-^{3}H (62 mg) at 2:00 PM.

Consider first the data obtained in period II, throughout which absorption was estimated by Method II. After the first 8 days of cholesterol feeding the results obtained by Methods II and IV were in very close agreement. However, in the first 8 days of period II the results obtained by Method II were markedly higher than all subsequent measurements by Methods II or IV; we consider this result by Method II to be falsely high, and due most likely to a lag in the intestinal transit of the first few days' intake of the new diet. It should be possible to minimize discrepancies of this sort by measuring accurately the transit time and first appearance of stools derived from any newly instituted diet; this was not done in this experiment.

Fig. 2 shows that in periods I and III (when the diet was free of cholesterol) the absorption of a single dose of 600 mg was considerably greater than in period II when the same dose of cholesterol was fed five times a day for many weeks. Thus, the total daily intake of cholesterol to which the patient had been conditioned affected the

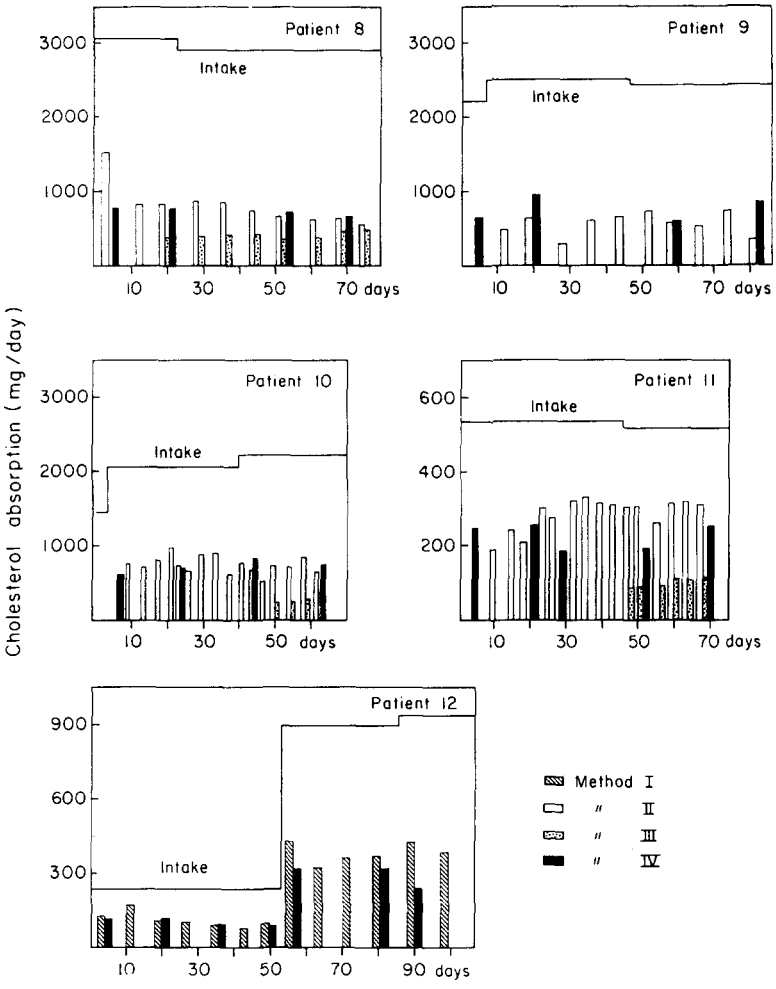

FIG. 1. Repeated measurements of cholesterol absorption by four methods in five patients.

absorption results; presumably this is related to the finding in rats that the size of the mucosal pool of cholesterol is dependent on the daily cholesterol intake (22). It seems clear that the size of the cholesterol dose used in any test by Method IV must take into account the prior intake of cholesterol in each patient tested; when tests are being made of the absorption of high intakes of cholesterol, the patient should be preconditioned to these intake levels before the test is begun. This was not done in Borgström's study (7).

III. Estimation of Cholesterol Absorption by Method III

We have noted (9) that, by definition, the calculation of cholesterol absorption by Method III is valid only during the isotopic steady state. Furthermore, we discussed the difficulties involved in recognizing the existence of this steady state simply by inspection of activity levels of plasma cholesterol. As an extension of that study, patients 8–11 were given daily oral intakes of radioactive cholesterol for periods of 44–86 days, and specific activities of plasma cholesterol were plotted against time (Fig. 3). In addition, sterol balance measurements were carried out throughout these periods, and the intake of radioactive cholesterol was compared to the total fecal excretion of radioactivity (after correction of neutral sterol losses through use of the internal standard β-sitosterol).

Although specific activities of plasma cholesterol appeared to reach a plateau in patients 8 and 11, in none of the four patients did the daily excretion of radioactivity equal the daily intake. Patient 8 appeared to reach a constant level of plasma specific activity after only 25 days of daily oral labeling, but at this time only about 82% of the isotope fed each day was being recovered in feces. During the next 50 days a gradual increase was observed in the fraction recovered, until at last the excretion of isotope reached the level of intake, but during this long period no significant changes were seen in levels of specific activity of plasma cholesterol. A different pattern was found in patient 9; in this patient, the recovery of isotope reached 95–90% after the first 12 days and remained rather constant thereafter for the next 74 days.

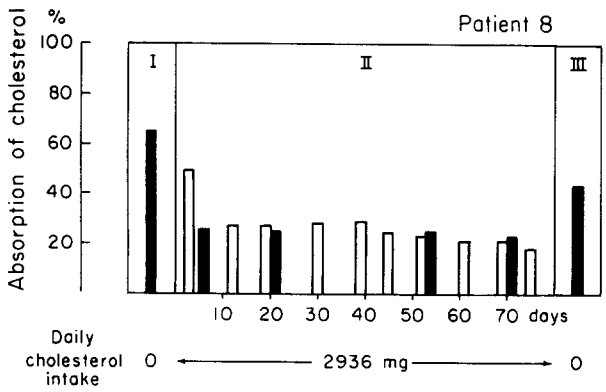

FIG. 2. Measurements of cholesterol absorption depend on the amount of cholesterol in the diet to which the patient has become adapted. The same test dose of cholesterol (600 mg) was administered in six measurements by Method IV (solid bars). When the patient was on a cholesterol-free diet, percentage absorption was greater than 44%, or in absolute terms greater than 260 mg; however, on a cholesterol-rich diet, percentage absorption was less than 30%, but in absolute terms about 730 mg. Comparability to results by Method II (open bars) was excellent so long as the same dietary conditions prevailed.

However, during this long period there was a gradual but progressive increase in specific activity of plasma cholesterol, a finding indicative of a persistent disequilibrium in labeling between plasma and tissues, and hence of a nonsteady state.

The results in patients 10 and 11 showed that, despite the feeding of radioactive cholesterol for long periods, the recovery of radioactivity in feces never approached the amounts fed, as if labeled cholesterol was continuously being taken into tissue pools. Indeed, since both patients had extensive xanthomatosis, it seems likely that radioactive cholesterol was exchanging slowly with large pools of sluggishly exchangeable cholesterol in xanthomas and in other tissues. A surgical biopsy of various superficial tissues was carried out in patient 11 after 67 days of daily oral intake of labeled cholesterol. As seen in Table 7, the specific activity of cholesterol in a tuberous xanthoma overlying one elbow was only 3% of that of the plasma; indeed, the specific activities in other tissues were also well below that of plasma cholesterol, and this in turn was only 14% of that of the diet (Fig. 3).

The results of these four studies show clearly that the attainment of a plateau of specific activity of plasma cholesterol is not per se a reliable guide for determining the attainment of an isotopic steady state. When the isotopic steady state has not been reached, i.e., when isotope excretion does not equal intake *and* plasma specific activities have not reached a plateau, measurements of cholesterol absorption by Method III will necessarily be underestimated. As shown in Fig. 1, the data obtained by Method III were consistently lower than those by the other methods. Nevertheless, in patients 8 and 11 the values for absorption calculated by Method III tended to increase throughout the period, and thus the differences between Methods II and III grew steadily smaller. If this changing state (expressed as the ratios of the results obtained by the two methods) is plotted sequentially along with the ratio of intake to excretion of isotope, it can be shown that both curves can be extrapolated to a ratio of unity at about the same time (Fig. 4). In principle, the time at which the two estimates become the same denotes the time at which the isotopic steady state is reached; in patient 8 we reason that this state would have been reached in about 94 days and in patient 11 between 120 and 140 days.

TABLE 7 FAILURE TO ATTAIN ISOTOPIC STEADY STATE IN A PATIENT (NO. 11) WITH HYPERCHOLESTEROLEMIA AND XANTHOMATOSIS*

Tissue Sampled†	Cholesterol Concentration	Specific Activity of Cholesterol‡
	mg/100 g wet wt	
Plasma	430	100
Muscle	68	78
Skin	147	66
Adipose tissue	118	45
Tuberous xanthoma	1970	3

* After 67 days of continuous oral administration of cholesterol-4-^{14}C (see Fig. 3), and 40 days after plateau of specific activity of plasma cholesterol had been reached.

† Muscle, adipose tissue, and overlying skin were biopsied in one deltoid region; a tuberous xanthoma overlying one elbow was resected.

‡ Relative to that in plasma.

DISCUSSION

Our exploration of the possible usefulness of Method IV was undertaken because in theory it seemed to offer a simpler and more rapid approach to the measurement of cholesterol absorption than the earlier methods we had tested. We were encouraged in pursuing this evaluation by the appearance in 1969 of Borgström's description of the same basic approach (7), especially since his conclusions were different from those we had previously reported with Methods I and II and from the conclusions of other workers (5, 6).

The main advantage of Method IV appeared to derive from the expectation that any difference in isotope ratio in feces from that in the test dose could be ascribed to absorption, and that the use of an essentially nonabsorbable internal standard (radioactive β-sitosterol) would permit the measurement of cholesterol absorption on casual samples of stools without the need for complete collections. Furthermore, any losses of the radioactive cholesterol during its intestinal transit would be correctable, since we had already shown that losses of β-sitosterol

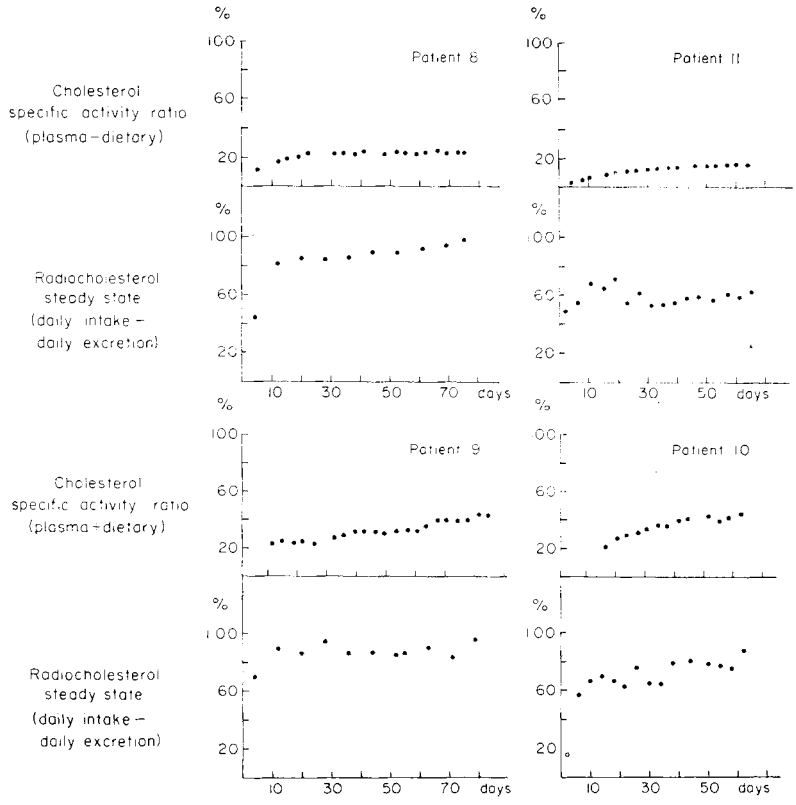

FIG. 3. Failure to reach the isotope steady state in four patients, as judged by two criteria: attainment of a plateau of specific activity of plasma cholesterol, and attainment of a balance between intake and excretion of orally administered radioactive cholesterol. The radioactive cholesterol steady state was not achieved in any patient, even though a plateau of radioactivity in plasma cholesterol seemed apparent in patients 8 and 11. In patient 11 the arrow signifies the time at which a surgical biopsy of various superficial tissues was carried out (see Table 7).

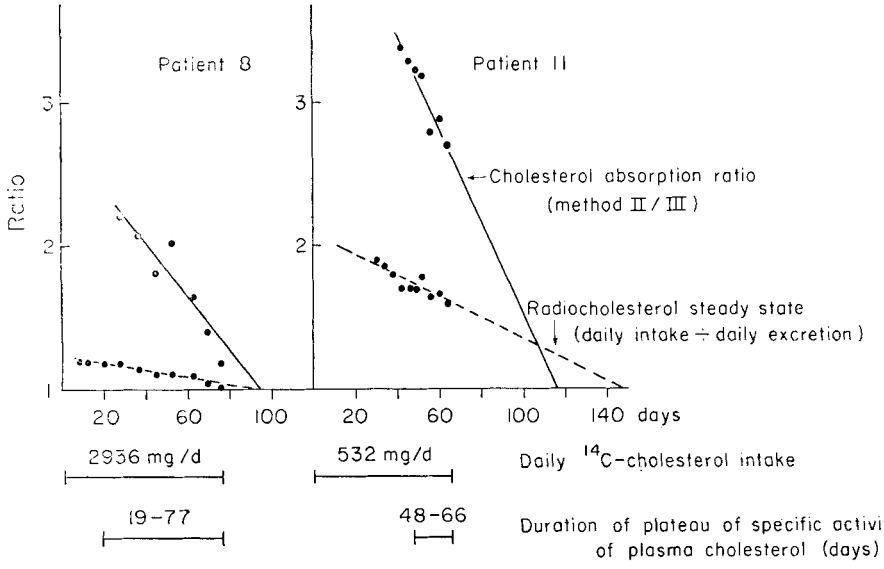

FIG. 4. Time required to attain the isotopic steady state, as predicted by approach to unity of two ratios: absorption by Method II ÷ absorption by Method III; and daily intake of labeled cholesterol ÷ its daily excretion. Note the disparities from unity for both ratios, even though an apparent plateau of specific activity of plasma cholesterol was reached in both patients (see also Fig. 3).

were of precisely the same magnitude (17). However, in our experimental validation of the method by analysis of successive stool specimens following oral administration of the test dose of radioactive sterols (Table 5), we were forced to the conclusion that any data derived from casual stool specimens would be misleading (due to temporary delays of radiocholesterol in the mucosa caused by isotope exchange), and that total stool collections were required for several days, or as long as significant excretion of radioactive sterols persisted.

Despite these findings, Method IV is the simplest and most rapid of all available methods for measurement of cholesterol absorption. It is a further advantage that only a very small amount of radioactivity is required: 1 μCi of each radiosterol is more than ample for one test by Method IV, whereas in our experience Methods I and II require at least 50 μCi of radioactive cholesterol (and for Method III as much as 200 μCi may be needed).

However, we believe the results shown in Fig. 1 prove that Method II is superior to all the others in precision and reproducibility, as well as being the most sound theoretically, for reasons we have previously discussed (9). 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 wk 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. These difficulties notwithstanding, it seems to us that Method II offers the best approach for meticulous studies of cholesterol absorption as a function of dietary cholesterol load.

Method I also has the advantage of simplicity, but there are drawbacks that we have previously described in detail (9). After intravenous administration of at least 50 μCi of radioactive cholesterol, calculations of absorption can be made almost immediately and thereafter repeatedly, 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 (19), or both, results by Method I will have doubtful validity.

Method III has the fewest advantages of all procedures we have examined, although it is theoretically sound provided that attainment of the isotopic steady state can be substantiated. We have shown here and previously (9) how difficult and time-consuming it may be to judge when this point has been reached. The time required can be shortened if a priming dose of radiocholesterol is administered at the start of the test (9); but even with the formulas of Zilversmit and Wentworth for selection of the optimal priming dose (23), several weeks will be needed to reach the isotopic steady state and to accumulate sufficient data to substantiate that conclusion. Kaplan et al. (5) have carried out many such studies in out-patients without testing rigorously whether a steady state has been reached, but in our experience Method III is feasible only when a patient can be committed to a long-term balance study on a metabolic ward, and when the following criteria for attainment of isotopic steady state are met: (*a*) an unchanging level of specific activity of plasma cholesterol, (*b*) proof that the amount of radioactive sterol fed each day is matched by the amount excreted (after correction of neutral sterol losses with dietary β-sitosterol as internal standard), and (*c*) correspondence between calculations of cholesterol absorption by Methods II and III (Fig. 4).

Methods I, II, and III share one disadvantage that has not yet been discussed, namely, the discrepancy in measurement of absorption that necessarily occurs if there is any appreciable contribution of nonlabeled cholesterol to the intestinal contents other than from the diet. For instance, if cholesterol synthesized in the liver or intestinal mucosa is excreted into the intestinal lumen before it reaches isotopic equilibrium with that in the plasma, the radioactivity of sterols in the lumen, and later in feces, will be diluted to some undefined degree. In animal studies, any sterols ingested through licking of fur will also dilute the radioactivity in the fecal neutral steroids. In both examples, the absorption of dietary cholesterol will be underestimated by Method I and overestimated by Methods II and III. Licking of fur is common in most laboratory animals, and excretion of cholesterol of specific activity significantly lower than that of plasma cholesterol has been noted by us in patients with a surgical bypass of the terminal ileum.[2] Therefore, in interpreting measurements of absorption made by Methods I, II, or III, the possibility should always be considered that special circumstances may produce a discrepancy between specific activities of plasma cholesterol and fecal endogenous neutral steroids. Method IV is not subject to this disadvantage; indeed, the results may be more reliable than any other in laboratory animals and, under certain conditions, in man.

[2] Grundy, S. M., and E. H. Ahrens, Jr. Unpublished results.

We now realize but have not yet explored a further advantage of Method IV that may make it the method of choice under many circumstances. When the investigator is interested in knowing the percentage absorption of cholesterol under any set metabolic conditions, that is, when the diet is unchanged prior to and during the test, an accurate estimate of cholesterol absorption should be obtainable by oral administration of the two isotopic sterols in a dose containing negligible mass. Prior to the test the mucosa will have reached an equilibrium for nonradioactive β-sitosterol and for cholesterol (exogenous or endogenous), and this equilibrium will not be upset by the test dose, since it will contain no mass of nonradioactive sterols. Collection of feces should be made continuously until the excretion of radioactive sterols becomes negligible. The difference in isotope ratio between test dose and that in fecal neutral steroids ought then to reflect the absorption of cholesterol. When the diet is cholesterol-free, this procedure ought to permit measurement of the absorption of recirculated cholesterol of endogenous origin.

For all methods, we stress that any losses of neutral steroids that may occur during their intestinal transit, whether these be mechanical (incomplete collections) or degradative, should be assessed and corrected through measurement of the recovery of a suitable internal standard; we have described our reasons for using dietary β-sitosterol as a marker for this purpose (17). With Method IV this correction is inherent in the calculation.

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