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Validation of the Ratio Method for Calculating Absorption of Dietary Cholesterol in Man[1] (37758)

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In 1970 we proposed a relatively simple method for calculating absorption of dietary cholesterol (1, 2). Unknown to us at that time, the same principle had been applied by Arnesjo *et al.* (3) to determine the site of cholesterol absorption in experimental animals. Although the principle of the method for calculating absorption of dietary cholesterol appeared valid (1–3), it had not been subjected adequately to experimental tests. The latter appeared all the more necessary in light of a suggestion in 1971 from Quintao *et al.* (4) that the differences between dietary cholesterol and β-sitosterol in their transit times through the GI tract were such that their ratio in a casual specimen of feces would give erroneously high values for absorption of dietary cholesterol. The following studies were, therefore, undertaken to test the validity of the ratio method proposed by us (1, 2).

Methods. Radioactive isotopes of cholesterol and β-sitosterol (Amersham/Searle) were purified by thin-layer chromatography before use. Seemingly healthy adult males were used as volunteers for these studies. Cholesterol-4-^{14}C and β-sitosterol-1-2-^{3}H were dissolved in corn oil, and known volumes of the oil were dispensed in gelatin capsules (2). From an aliquot of the oil containing radioisotopes, the quantities and the ratios of the two isotopes administered to the experimental subjects were determined. The subjects were given a standard breakfast of one egg, two pieces of toast, butter, jam, and coffee. The capsule containing the radioisotopes was ingested just before the breakfast was finished. Another capsule containing about 500 mg of carmine red marker was also taken with the breakfast. The subjects were asked to follow their usual routine in eating and other activities. They were given empty plastic (ice cream) containers and an aluminum sheet through which a hole was cut to hold the containers. The aluminum sheet and the containers fitted in the toilet bowl so that feces could be collected conveniently. The subjects were asked to collect each fecal specimen in a separate container, to close the tight-fitting lid, and to bring all the specimens to the laboratory. The fecal neutral steroids were extracted by the method of Miettinen *et al.* (5). The specimen containing the maximum of red color was selected to calculate the absorption of dietary cholesterol. Total quantities of radioactive cholesterol and β-sitosterol in the entire 8-day fecal specimens were also determined to calculate the absorption by Borgstrom's method (6). Losses were corrected by data on recovery of radioactive β-sitosterol (7).

Male rats of Sprague–Dawley strain were used for experimental studies. The radioisotopes were given by gastric intubation. The details of bile duct cannulation and cannulation for infusion of normal rat bile were as those described by us recently (8). The animals were kept in restraining cages (8), and the feces were collected on filter paper. The animals were anesthetized with ether and exsanguinated by withdrawing blood from the abdominal aorta. The intestinal contents were flushed with physiological saline and collected. The heads of the animals were re-

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moved, and the remaining carcass was cut into small pieces and put in alcoholic kOH for a few days; and the nonsaponifiable fraction was extracted with petroleum ether (9). The radioactivity was determined in a Packard Liquid Scintillation Spectrometer (10).

Results. In order to investigate the difference in the transit time of cholesterol and β-sitosterol, three rats were given a mixture of cholesterol-1-2-^{3}H and β-sitosterol-4-^{14}C (ratio:6.6) by gastric intubation. Three hours later the animals were anesthetized, and duplicate ligatures were applied to divide the small intestine into four segments and the large intestine into two equal segments. The intestines were then divided between the ligatures, and the ratios of the two isotopes in intestinal contents were determined. The ratio in the most proximal intestinal segment was 14.5 ± 2.2, and it progressively decreased to 1.3 ± 0.4 in the most distal segment of the large intestine. The ratios in the first two proximal segments were greater than even the ratio of cholesterol-^{3}H/β-sitosterol-^{14}C administered (Fig. 1).

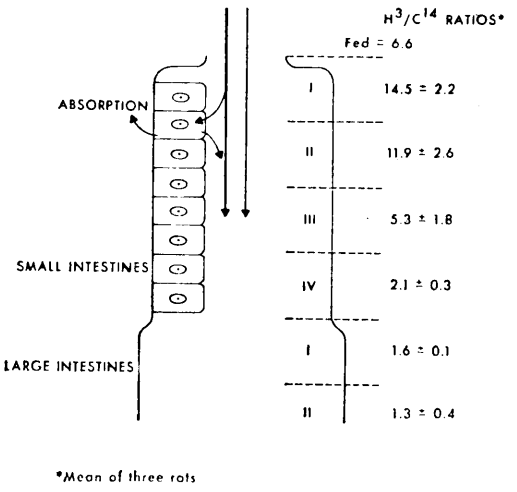

FIG. 1. Changes in the ratios of cholesterol-^{3}H and β-sitosterol-^{14}C in intestinal contents after their gastric intubation in three rats.

To see if this phenomenon (whatever the explanation) influenced the ratios of the two isotopes in specimens of feces collected in pools of many hours, six rats were given the mixture of radioactive sterols by gastric intubation. In order to avoid the absorbed radioactive cholesterol from contaminating the intestinal contents, the bile ducts of these animals had been cannulated. Fresh bile from donor rats was infused continuously through another cannula in the duodenum in half of these animals (shown on the right in Fig. 2). The ratios of the two isotopes in the fecal specimens collected at different intervals failed to show any progressive change in five out of six animals. Despite variations in the excretory patterns of the neutral steroids in different animals, the percentage of excretions in each specimen were identical for cholesterol and β-sitosterol (Fig. 2). These data indicated that the "lumen–mucosa–lumen" cycle took such a short time that its effects on the fecal samples collected over many hours

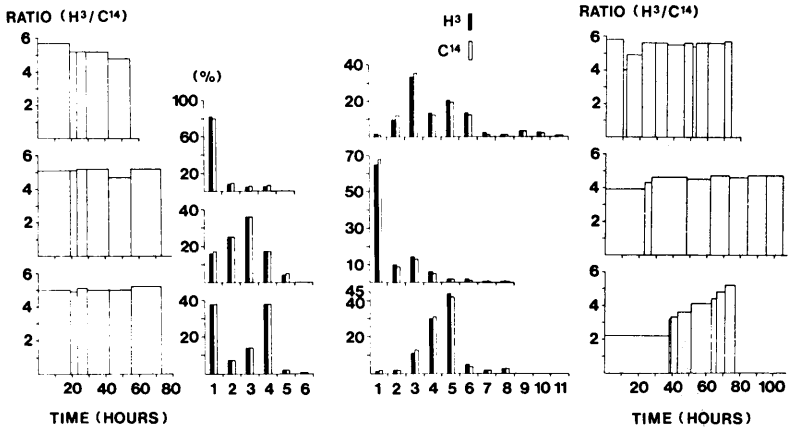

FIG. 2. Percentage recovery and ^{3}H/^{14}C ratio in sequential samples of fecal neutral steroids after duodenal intubation of cholesterol-^{3}H and β-sitosterol in rats with bile duct fistulae. Three rats (shown on the right) had intraduodenal perfusion of normal rat bile and the three (shown on the left) did not.

TABLE I. Comparison of the Ratio Method for Calculating Absorption of Dietary Cholesterol with the Actual Absorption Determined in Rats.[a]

Rat number	Cholesterol fed (mg)	Percent absorption: Actually determined (1)	Percent absorption: Ratio method (2)	Difference (2)—(1)	Cholesterol absorbed (mg) Ratio method
1	0.0	83.8	79.6	—4.2	0
2	0.0	85.2	77.9	—7.3	0
3	1.0	75.2	77.2	+2.0	0.77
4	1.0	80.0	91.8	+11.8	0.92
5	1.0	88.6	92.9	+4.3	0.93
6	1.6	82.7	71.5	—11.2	1.14
7	1.6	75.1	73.9	—1.2	1.18
8	2.0	65.1	79.1	+14.0	1.58
9	2.0	53.6	62.0	+8.4	1.24
10	2.0	75.2	82.0	+6.8	1.64
11	2.7	70.9	78.4	+7.5	2.11
12	2.7	70.5	71.1	+0.6	1.92
13	4.0	42.0	48.0	+6.0	1.92
14	4.0	32.5	34.0	+1.5	1.36
15	4.0	33.4	43.1	+9.7	1.72
16	5.1	37.1	35.5	—1.6	1.82
17	5.1	36.2	40.3	+3.6	2.05
18	6.0	30.8	40.0	+9.2	2.40
19	6.0	37.2	35.4	—1.8	2.12

[a] $r = 0.95$. Mean ± SD, $+3 \pm 7$.

were not apparent in five out of six animals.

In order to check the results calculated by the ratio method against actual values of absorption, 19 rats were given a mixture of cholesterol-1-2-^{3}H and β-sitosterol-4-^{14}C along with different amounts of carrier cholesterol (Table I). The fecal samples containing the unabsorbed sterols were collected and the absorption calculated by the ratio method (2). The contents of the intestinal tract were removed and the total radioactivity in the carcass of each animal was determined as percentage of the total administered dose (Table I). The values obtained by the two methods were generally similar, and they had excellent correlation ($r = 0.95$). The values obtained by the ratio method were $3 \pm 7\%$ greater than those actually determined. The range of the difference between the two methods was between —7.3 and +14.0%. In the absence of any carrier cholesterol in the intubated mixture, the percentage of absorption was greater than 80, and the percentage absorption progressively decreased as the amount of carrier cholesterol was increased in the intubated mixture. The absorption with 6.0 mg of dietary cholesterol was only about 30%. The amounts of absorbed cholesterol, however, tended to increase with increment in intake of cholesterol.

To compare results obtained by the ratio method with those obtained by Borgstrom's method, 20 human volunteers were given a mixture of cholesterol-1-2-^{3}H and β-sitosterol-4-^{14}C along with carmine red as a marker. Fecal samples were collected individually, and their color was noted. The ratios of the two isotopes in the samples containing the greatest amount of red dye were determined, and the percentage absorption was calculated as per methods published previously (2). The total recovery of cholesterol and β-sitosterol in fecal samples for 8 days was also determined, and the absorption was calculated by Borgstrom's method (6). The results (Table II) indicated that the values ob-

TABLE II. Comparison of the Ratio Method with Borgstrom's Method for Calculating Absorption of Dietary Cholesterol in Man.[a]

Subject number	Percent absorption: Borgstrom's method (1)	Ratio method (2)	Difference (2)—(1)
1	49.2	55.0	+5.8
2	49.0	50.0	+1.0
3	54.5	53.8	—0.7
4	42.7	43.3	+0.6
5	62.1	65.9	+3.8
6	66.4	62.8	—3.6
7	47.0	54.3	+7.3
8	40.3	43.3	+3.0
9	58.0	58.0	0.0
10	70.5	68.5	—2.0
11	47.3	48.6	+1.3
12	59.7	61.9	+2.2
13	50.0	52.0	+2.0
14	63.7	71.0	+7.3
15	64.8	62.8	—2.0
16	55.4	55.8	+0.4
17	50.0	54.9	+4.9
18	50.0	50.0	0.0
19	54.5	67.7	+13.2
20	61.4	65.9	+4.5

[a] $r = 0.89$. Mean ± SD, +2 ± 4.

tained by both methods were similar; the values obtained by the ratio method were on the average 2% higher than those obtained by Borgstrom's method. The correlation between the results obtained by the two methods was excellent ($r = 0.89$). The ranges of values for absorption were between 43.3 and 71.0% for the ratio method and between 40.3 and 70.5% for Borgstrom's method.

Discussion. The use of a nonabsorbable marker to calculate the absorption of cholesterol (11) appears sound so long as the marker has the same transit time in the intestinal tract as that of cholesterol. However, dietary cholesterol may be degraded in the intestinal tract; and since the degradation varies considerably in different individuals, it is necessary that the marker should also share this property equally with cholesterol (7). Although the question of degradation of dietary sterols is still unsettled (12), it is desirable to use β-sitosterol (2) as a marker rather than chromic oxide (11).

After simultaneous administration of cholesterol-^{14}C and β-sitosterol-^{3}H to a number of subjects, Quintao *et al.* observed progressive increase in $^{14}C/^{3}H$ ratios in sequential fecal samples (4). We have also observed a similar increase in the ratios in sequential fecal samples in subjects who had intact biliary system (13). Quintao *et al.* attributed this to "back exchange" of radioactive cholesterol in the intestinal mucosa with nonradioactive dietary cholesterol in the intestinal lumen. In patients with obstructive liver disease (4) or bile fistula (13), however, this progressive increase in the ratios was not seen. The fecal $^{3}H/^{14}C$ ratios remained constant. Experimental studies on rats with bile duct fistulas also indicated that the $^{3}H/^{14}C$ ratios in fecal neutral steroids remained constant in (five out of six) animals given cholesterol-^{3}H/β-sitosterol-^{14}C. Absence of change in the $^{3}H/^{14}C$ ratios in obstructive liver disease was attributed by Quintao *et al.* (4) to lack of bile salts and thus of solubilization of dietary cholesterol, which is necessary for isotopic exchange. However, we are inclined to think that increment in the ratios of fecal isotopes in man or in experimental animals with intact biliary systems is more likely to be due to progressively increasing relative contributions of biliary cholesterol to the fecal radioactivity than to isotopic exchange. Absence of change in fecal ratios, even in animals given bile infusion, is in accord with this suggestion. The unusual observation in one of the six rats may be due to the presence of an accessory bile duct in that animal; however, there is no other evidence for that.

Although our observations on the cholesterol-$^{14}C/$β-sitosterol-^{3}H ratios in the proximal intestines of rats are in accord with the isotopic exchange hypothesis of Quintao *et al.* (4), another explanation may be equally likely: Radioactive cholesterol was taken up by the intestinal wall; a fraction of it was absorbed into the intestinal lymph, and the remainder was released back into the lumen. During the time the cholesterol was going through this "lumen–mucosa–lumen" cycle at the same level of the intestinal tract, the

unabsorbed dietary sterols had moved down along the length of the intestines. The mechanism of the release of cholesterol from the intestinal wall into the lumen is not quite clear at the moment: It may or may not be related to exfoliation of absorptive cells on the tips of villi. Release into the lumen has also been observed with regard to the cholesterol synthesized in the intestinal wall (14). Since the presence or absence of cholesterol in the intestinal lumen did not make any difference to the amount of cholesterol released from the intestinal wall (14), isotopic exchange becomes an unlikely explanation under those conditions. Furthermore, it appears that once the cholesterol is released from the intestinal wall into the lumen it is not as readily absorbed as the dietary cholesterol (14), which is more in favor of the exfoliation hypothesis than that of isotopic exchange.

It has been shown that the results obtained by Borgstrom's method are nearly the same as those obtained by other more reliable methods developed at the Rockefeller University (4). Our studies indicate that the results obtained by the ratio method were also similar to those obtained by Borgstrom's method provided the fecal specimen containing most of the unabsorbed dietary sterols and the red marker were taken for analysis. This represents a modification of the method proposed earlier (2). In the earlier version it was suggested that the very first specimen containing radioisotopes should be analyzed.

The critical proof for the validity of this method (at least in rats) is given by the actual determination of total radioactivity in the carcass of the animals given the isotopes by gastric intubation. The percentage absorption calculated (by the ratio method) from the isotopes in the feces collected over the first 24 hrs was in remarkable agreement with the values determined experimentally, although the values for absorption actually determined appeared to have less scatter than those calculated by the ratio method. The correlation between the values obtained by the two methods was excellent ($r = 0.95$).

Summary. In 1970 we proposed a simple method for calculating absorption of dietary cholesterol in man. Although the principle of the method appeared quite valid, the method itself has not been subjected to adequate experimental test. Studies were, therefore, undertaken to test the validity of the method. Our results suggest that a fraction of dietary cholesterol in the intestinal mucosa is released back into the intestinal lumen. The time taken for this "lumen–mucosa–lumen" cycle is so short that its effects were not seen in rat fecal pools collected over many hours.

The values of absorption of dietary cholesterol in man calculated by our method were similar to the values obtained by Borgstrom's method. The critical evidence for the validity of the method was provided by experimental studies in rats. The calculated values for percentage absorption were in excellent agreement with those actually determined in the rat carcasses.

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