papers on methodology

Reevaluation and application of the dual-isotope plasma ratio method for the measurement of intestinal cholesterol absorption in the hamster

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Abstract These experiments systematically evaluated the dual-isotope plasma ratio method for measuring intestinal cholesterol absorption in the hamster. It was found that while the ratio of the ^{3}H- and ^{14}C-labeled cholesterol in the plasma, relative to the respective dose of each that was given, became constant by 72 h after their administration, the percent cholesterol absorption was lower in animals that were fasted before dosing (35.7 ± 5.5%) than in their fed controls (47.5 ± 3.7%). Furthermore, the percent absorption found 72 h after dosing varied greatly, depending on whether the intragastric dose of labeled cholesterol was administered in medium chain triglyceride (MCT) oil (46.2 ± 2.3%), olive oil (63.9 ± 11.2%), or safflower oil (74.6 ± 4.5%). The level of absorption was not different between hamsters that had unrestricted (46.3 ± 1.6%) and restricted (43.8 ± 2.2%) access to their stools during the 72 h after dosing. Other experiments, using only hamsters in the fed state and MCT oil as the intragastric dosing medium, showed that the percent cholesterol absorption could be made to vary over a wide range using treatments known to produce such effects in humans. Thus, feeding either surfomer, cholestyramine, ursodeoxycholic acid, or CI-976, a new inhibitor of acyl-CoA:cholesterol acyltransferase, significantly blocked cholesterol absorption, whereas the addition of either cholic acid or increasing amounts of oil to the diet had the opposite effect. The modified dual-isotope plasma ratio method described here provides a simpler and more physiologic approach to the routine measurement of cholesterol absorption in the hamster and similar small animal models.—**Turley, S. D., M. W. Herndon, and J. M. Dietschy.** Reevaluation and application of the dual-isotope plasma ratio method for the measurement of intestinal cholesterol absorption in the hamster. *J. Lipid Res.* 1994. **35:** 328–339.

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It is now well accepted that there is a strong association between the development of arteriosclerosis and the level of cholesterol carried in the plasma low density lipoproteins (LDL) (1). Like the cholesterol present throughout the remainder of the body, that carried in LDL is derived ultimately from either de novo cholesterol synthesis in the tissues or from dietary cholesterol that is absorbed across the wall of the small intestine (2). Studies in humans have shown that there is an overall correlation between plasma LDL-cholesterol concentrations and the efficiency of intestinal cholesterol absorption (3, 4). There are several techniques for measuring cholesterol absorption, and these have been the subject of extensive evaluation and review (5–8). One of these is the dual-isotope plasma ratio method, variations of which have been applied in many species (7, 9–17). There are, however, no published reports of this technique being used in the hamster, although other methods have been applied to a limited extent in this species (18–21).

The lack of published data on the measurement of cholesterol absorption in the hamster is in marked contrast to the detailed information that exists concerning other features of its sterol metabolism. Thus, there is now an extensive literature describing the basal rates of cholesterol synthesis and LDL clearance by the liver and extrahepatic organs, as well as the composition of the bile acid pool, and the profile of plasma lipoproteins in this species (22–27). In addition, the structure of the gene for the LDL receptor in this animal model has been elucidated (28). The hamster has now become one of the most widely used species for studying the mechanism by which fatty acids and other dietary components exert their regulatory effects on plasma LDL-cholesterol levels (29–32), as well as for evaluating the efficacy of a diverse range of new cholesterol lowering agents (18–21, 33–36).

As none of the techniques for measuring intestinal cholesterol absorption have been systematically tested in the hamster, the present experiments were carried out to

Abbreviations: MCT, medium chain triglyceride; UDCA, ursodeoxycholic acid; CA, cholic acid; ACAT, acyl-CoA:cholesterol acyltransferase; EDTA, ethylenediamine tetraacetic acid; LDL, low density lipoprotein.

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evaluate the dual-isotope plasma ratio method in this species. These experiments focused on several aspects of the technique that could be either simplified or modified to yield a more physiologically meaningful measure of the efficiency of cholesterol absorption in animals under specific experimental conditions. In the past, the intravenous dose of labeled cholesterol was administered in an aqueous suspension, while the intragastric dose was given in an oil phase containing significant quantities of added cholesterol, bile acid, and other ingredients. Furthermore, the experimental subjects were usually fasted for an extensive period before and after dosing. These conditions are less than optimal for obtaining the best measure of the effect of a particular dietary or pharmacologic manipulation on cholesterol absorption because the intragastric dose of labeled cholesterol is introduced into a gastrointestinal tract devoid of the test agent or diet that is being evaluated.

In this paper we thus describe a modification of the dual-isotope plasma ratio method and its application to the measurement of cholesterol absorption in hamsters subjected to an array of experimental manipulations known to change the efficiency of absorption in humans. A standard protocol was established in which non-fasted animals are dosed intravenously with [^{3}H]cholesterol contained in Intralipid and intragastrically with [^{14}C]cholesterol mixed in MCT oil. The animals then continue to receive their respective experimental treatment for a further 72 h, at which point the level of cholesterol absorption is determined directly from the ratio of the proportion of the [^{14}C]- and [^{3}H]cholesterol doses remaining in the plasma at that time.

MATERIALS AND METHODS

Animals and diets

Male, outbred, virus-free Golden Syrian hamsters (*Mesocricetus auratus*) weighing 80–100 g were obtained from Charles River Laboratories (Montreal, Canada). The hamsters were housed in plastic colony cages containing wood shavings in a room with alternating periods of light (1:00 PM to 1:00 AM) and dark (1:00 AM to 1:00 PM). They had free access to water at all times. During an initial adaptation period of 2 weeks, all hamsters were fed ad libitum a plain pelleted chow diet (No 7001, Teklad Premier Laboratory Diets, Madison, WI). All the experimental diets were prepared with the meal form of another blend of plain rodent chow (Wayne Rodent Blox No 8604) (also supplied by Teklad Premier Laboratory Diets). The Wayne chow diet had a proximate analysis of: crude protein 24% (min), crude fat 4.0% (min), and crude fiber 4.5% (max). This diet also had a basal cholesterol content of 0.024% (wt/wt) and a fatty acid composition as follows (expressed as a percent of total fat): linoleic acid (46.1%), oleic acid (21.7%), palmitic acid (19.5%), linolenic acid (4.6%), stearic acid (4.4%), palmitoleic acid (2.2%), and myristic acid (1.5%). The ground Wayne chow was used to prepare a series of different experimental diets. For one experiment, cholesterol (Byron Chemical Company, Long Island City, NY) was added to the chow at levels of 0.12, 0.24, and 0.50% (wt/wt). An additional lot of the diet with 0.12% added cholesterol was also made to contain CI-976 (Parke-Davis, Ann Arbor, MI), a newly developed ACAT inhibitor, at a level of 0.12%. In other experiments the chow diet containing added cholesterol (0.12%) was also made to contain either cholic acid (Sigma Chemical Company, St. Louis, MO) or ursodeoxycholic acid (Sigma), each at a level of 0.10%, or safflower oil, at a level of either 10% or 20%. In separate studies, diets were prepared that contained added cholesterol (0.12%), hydrogenated coconut oil (ICN Biomedicals, Irvine, CA) (10%), and either cholestyramine (Bristol-Myers Squibb Company, Evansville, IN) or surfomer (Monsanto Company, St. Louis, MO), each at levels of 0, 1.0, 2.0, 3.0, and 4.0%. Unless otherwise indicated, all hamsters were fed their respective diets ad libitum for 10 days. Body weights were recorded at the beginning and end of each experiment. In the study that was designed to test whether the level of cholesterol absorption differed in hamsters that had either restricted or unrestricted access to their stools after dosing, the animals were housed singly in cages that were fitted with either a wire mesh floor only, or a wire mesh floor placed over a deep layer of wood shavings. Thus while all the hamsters had to contend with single rather than group housing and with a wire floor surface, one group continued to have access to their stools while the other did not.

Preparation of labeled cholesterol mixtures

As described in detail below, each hamster received an intravenous dose of about 2.5 μCi of [1,2-^{3}H]cholesterol (Amersham Corp, Arlington Heights, IL) contained in Intralipid (20%) (Kabi Pharmacia Inc., Clayton, NC), followed by an intragastric dose of about 1.0 μCi of [4-^{14}C]cholesterol (Amersham) contained in MCT oil (Mead Johnson & Company, Evansville, IN). For each experiment the required total activity of [^{3}H]cholesterol was taken to dryness under nitrogen in a glass tube, and then redissolved in absolute ethanol (2 μl ethanol per μCi of ^{3}H). To this solution was then added undiluted Intralipid (0.16 ml Intralipid per μCi of ^{3}H). This mixture was vortexed vigorously for 3 min. The [^{14}C]cholesterol, after being taken to dryness under nitrogen, was also redissolved in ethanol (10 μl per μCi of ^{14}C). To this solution was then added MCT oil (0.60 ml per μCi of ^{14}C). The addition of the MCT oil was carried out in two stages. Initially, 5 ml of MCT oil was added to the [^{14}C]cholesterol in ethanol. After mixing vigorously for 3 min on a vortexer, the remaining portion of MCT oil was then added and the tube was mixed for a further 3 min. In one experi-

ment the MCT oil was substituted with either olive oil or safflower oil to test whether the level of cholesterol absorption varied with the type of medium that was used for the intragastric administration of the [^{14}C]cholesterol. To check that the labeled cholesterol was uniformly mixed in the Intralipid and MCT oil, 10 μl aliquots of each mixture were added directly to 10 ml of Ecolite (ICN Biomedicals, Inc., Irvine CA) and counted in a liquid scintillation spectrometer (Packard Instrument Company, Downers Grove, IL). The ^{3}H- and ^{14}C-labeled mixtures were prepared fresh on the day of each experiment, and both were kept at room temperature during and after preparation. For each study about 15% more of each labeled mixture was prepared than was needed for dosing all the animals. A portion of this excess material was used for the preparation of standards that were required to determine the actual doses of [^{3}H]- and [^{14}C]cholesterol that were administered.

No attempt was made to further purify the batches of [^{3}H]- and [^{14}C]cholesterol before they were mixed with Intralipid or MCT oil. However, the particular batches of both the [^{3}H]- and [^{14}C]cholesterol that were used in most of the experiments described here were screened in vivo by a protocol described by other investigators, who in the past, had shown that some batches of [^{3}H]cholesterol were radiochemically unreliable (37).

Intravenous and intragastric administration of labeled cholesterol mixtures

Except in one experiment with fasted animals, the hamsters always had free access to their diets up until the time of dosing, which was carried out within about the first 4 h after the onset of the light period (i.e., between 1:00 and 5:00 PM). The hamsters were anesthetized lightly with diethyl ether and an incision of about 4 cm was made in the groin of the left leg directly above the femoral vein. The dose of [^{3}H]cholesterol in Intralipid (0.40 ml) was then injected directly into the distal tip of the femoral vein using a calibrated plastic tuberculin syringe (1.0 ml) fitted with a 30G needle (1.27 cm in length). This injection was done slowly over 30 to 45 sec so as to prevent any backbleeding after withdrawal of the needle. The incision was then tightly closed with 4-0 silk sutures. Each hamster was then immediately administered i.g. the required dose of [^{14}C]cholesterol in 0.60 ml of MCT oil. This was done using a calibrated Glaspack syringe (1.0 ml) fitted with a blunted 18G (3.8 cm) needle to which was attached a 12-cm section of PE 190 polyethylene tubing (Clay Adams, Parsippany, NJ). The intravenous and intragastric dosing of each hamster was routinely completed within 7–8 min. After recovering from the anesthesia (15–20 min), the hamsters were returned to their colony cages where food was immediately available to them. In the experiment designed to test whether the percent cholesterol absorption varied if the hamsters could not readily practice coprophagy, the animals were housed individually after they were dosed. With this protocol no problems were encountered with the hamsters regurgitating any of their gastric contents during and after the recovery period. They continued to receive their usual diet for the next 3 days during which time they either maintained or increased their body weight.

Two points concerning the use of Intralipid as a vehicle for the i.v. dosing of the [^{3}H]cholesterol should be emphasized. First, as Intralipid, at a strength of 20%, has a cholesterol concentration of only 0.30 mg/ml, the administration of 0.40 ml of the emulsion introduces a negligible quantity of exogenous cholesterol into the plasma compartment. Second, the labeled Intralipid mixture is cleared rapidly from the circulation. Although the data are not shown, it was found that at least 80% of the dose of [^{3}H]cholesterol was removed from the plasma within the first hour after injection into a femoral vein.

Determination of percent cholesterol absorption

Seventy two hours after dosing, the hamsters were again lightly anesthetized with diethyl ether and bled from the abdominal aorta into a syringe containing EDTA as anticoagulant. The liver was removed, rinsed, and weighed. Aliquots of plasma and liver were taken for the measurement of total cholesterol concentration. To determine the proportion of the doses of [^{3}H]- and [^{14}C]cholesterol remaining in the plasma after 72 h, duplicate 100 μl aliquots of plasma and of the original dosing mixtures were added directly to 10 ml Ecolite. The vials were shaken vigorously and left to stand under a fluorescent light overnight before being counted in a liquid scintillation spectrometer in the presence of an external standard to determine the level of quenching. This method thus provided a quick and simple means of accurately determining the levels of [^{3}H]- and [^{14}C]cholesterol in the plasma.

The data for the level of ^{3}H and ^{14}C activity in the samples and standards were used to calculate the percent cholesterol absorption using the following expression (9).

$$\text{Percent cholesterol absorption} = \frac{\text{Percent of i.g. dose ([}^{14}\text{C]cholesterol) per ml plasma}}{\text{Percent of i.v. dose ([}^{3}\text{H]cholesterol) per ml plasma}} \times 100$$

Determination of plasma, hepatic, and dietary cholesterol levels, and dietary fatty acid composition

Plasma total cholesterol concentrations were measured enzymatically using a kit (No. 1127578) from Boehringer Mannheim (Indianapolis, IN). Aliquots of liver were saponified and extracted, and the cholesterol concentration was measured by gas–liquid chromatography (GLC) using stigmastanol (Sigma) as an internal standard. Ali-

quots of diet to which [^{14}C]cholesterol had been added were extracted in chloroform–methanol 2:1 (vol/vol) and filtered. The extracts were dried, saponified in alcoholic KOH, and the sterols were then extracted in petroleum ether. The concentration of cholesterol in the petroleum ether phase was then measured by GLC using 5-cholestene (Sigma) as an internal standard. Separate aliquots of the organic phase were taken to determine the recovery of the [^{14}C]cholesterol. The quantitation of cholesterol by GLC was carried out using a model 5890 Series II instrument (Hewlett-Packard Company, Avondale, PA) fitted with a fused silica capillary column (HP-5) (length 25 m; internal diameter 0.2 mm; film thickness 0.33 μm) that was run at 315°C. The analysis of the fatty acid composition of diet extracts was carried out by GLC after methyl esterification of the fatty acids as described elsewhere (32).

Analysis of data

Where appropriate, means ± 1 SEM for groups of data are given. For correlating hepatic cholesterol concentrations with the percent cholesterol absorption, or the percent absorption with body weight, a linear regression line with the form y = a + bx was fitted by the method of least squares to the data from individual animals. Differences between mean values for the treated groups and their respective control were tested for statistical significance using the two-tailed unpaired Student's *t*-test.

RESULTS

To obtain a valid measure of intestinal cholesterol absorption by the dual-isotope plasma ratio method, it is essential to first determine the time required after dosing for the ratio of the proportion of the intragastric dose of labeled cholesterol remaining in the plasma to the proportion of the intravenous dose remaining to reach a constant value. The objective of the first experiment was to determine not only when this occurred in the hamster, but also to investigate whether the time required to reach a constant ratio, as well as the percent cholesterol absorption, varied depending on whether the animals were in a fed or fasted state at the time of dosing. For this experiment, all the hamsters were given a chow diet containing added cholesterol (0.12% wt/wt). The data in **Table 1** show both the dpm of each radiolabel per ml of plasma and the percent cholesterol absorption at 24, 48, and 72 h in hamsters that had been either fed ad lib or fasted for 6 h before and for 2 h after dosing. It is evident that, although the plasma ratio of the [^{14}C]cholesterol (given i.g.) to the [^{3}H]cholesterol (given i.v.) had stabilized by 72 h in both the fed and fasted groups, the apparent level of cholesterol absorption was consistently about 25% lower in the fasted animals than in the matching fed group. While this difference was not statistically significant ($P > 0.05$), the

TABLE 1. Comparison of the percent cholesterol absorption at various time points in male hamsters that were either fed or fasted before and after dosing with the radiolabeled cholesterol

Diet	Treatment Before and After Dosing	Final Body Weight	Level of Activity of Each Radiolabel in Plasma						Percent Cholesterol Absorption			
			^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H				
			24 h		48 h		72 h		24 h		48 h	72 h
		g	*dpm/ml plasma* × 10^{-3}						%			
A. Chow + cholesterol (0.12%)	Fed	151 ± 2	10.8 ± 1.1	73.2 ± 2.8	8.7 ± 0.8	50.1 ± 1.6	6.4 ± 0.5	35.1 ± 1.0	39.3 ± 3.6		45.6 ± 3.6	47.5 ± 3.7
B. Chow + cholesterol (0.12%)	Fasted	158 ± 3	7.5 ± 1.1	69.8 ± 3.1	6.4 ± 0.9	49.3 ± 2.2	4.8 ± 0.8	34.8 ± 1.7	28.4 ± 4.2		34.3 ± 5.0	35.7 ± 5.5

Male, virus-free hamsters were fed a chow diet containing added cholesterol (0.12% wt/wt) for a total of 10 days. On the seventh day the diet was withdrawn from one group of animals 6 h before they, and a matching fed group, were given an intragastric dose of [^{14}C]cholesterol (in MCT oil) and an intravenous dose of [^{3}H]cholesterol (in Intralipid). Food was withheld from the fasted group for a further 2 h after dosing. Both groups were then fed ad lib for the next 3 days during which time blood samples were taken from each animal at 24, 48, and 72 h after dosing. This was done by drawing about 0.5 ml of blood from a subclavian vein under light ether anesthesia. Aliquots of plasma from each time point were counted to determine its content of ^{3}H and ^{14}C, and these data were used to calculate the percent cholesterol absorption according to the dual-isotope plasma ratio method. Values are the mean ± 1 SEM of data obtained from eight animals in each group.

period of fasting before dosing (6 h) applied in this experiment was much shorter than that which has been routinely used with this method in the past (9, 11–13). From Table 1 it should be noted also that while only 2–6% of the initial dose of [^{3}H]- and [^{14}C]cholesterol remained in the plasma after 72 h, there were readily detectable levels of both isotopic labels in the aliquots of plasma that were assayed.

As a further validation of the technique, an experiment was done to determine whether the level of cholesterol absorption differed when hamsters were housed individually with or without access to their stools during the 72 h after dosing. A total of 22 hamsters fed the chow diet containing added cholesterol (0.12%, wt/wt) were dosed and then housed individually in cages fitted with either wire mesh floors only or mesh floors laid over wood shavings. The percent cholesterol absorption in the animals with restricted access to their stools (43.8 ± 2.2%, n = 11) did not differ significantly from that in the group that was unrestricted (46.3 ± 1.6%, n = 11). The plasma total cholesterol concentrations in these groups were 208.1 ± 5.9 and 211.0 ± 3.4 mg/dl, respectively.

The next experiment was designed to investigate whether the level of cholesterol absorption varied with the type of medium that was used for the intragastric administration of the [^{14}C]cholesterol. Groups of hamsters that had been given the chow diet containing added cholesterol (0.12%) all received an i.v. injection of [^{3}H]cholesterol in Intralipid, followed by an i.g. bolus of [^{14}C]cholesterol that was contained in either MCT oil, olive oil, or safflower oil. All of the hamsters were in the fed state at the time of dosing. The level of cholesterol absorption, calculated from the plasma ratio of ^{14}C and ^{3}H determined 72 h after dosing, is given in **Fig. 1.** A different level of absorption was found with every group. The lowest level was in the animals dosed with MCT oil (46.2 ± 2.3%), followed by the groups dosed with olive oil (63.9 ± 11.2%) and safflower oil (74.6 ± 4.5%). From the results of these initial experiments, a fixed protocol for measuring cholesterol absorption in the hamster was established. Thus, in all subsequent experiments, the hamsters were group-housed and were dosed only in the fed state, the intravenous and intragastric doses of labeled cholesterol were given in Intralipid and MCT oil, respectively, and the percent absorption was determined at 72 h after dosing.

The next group of studies was designed to test the dependability of this protocol by applying it to hamsters that had been subjected to a diverse selection of dietary and pharmacologic treatments known to change the efficiency of intestinal cholesterol absorption in humans. Specifically, six different manipulations were used. The first one examined the effect of varying the level of cholesterol in the diet over about a 20-fold range. Thus, hamsters were fed ad lib either the basal control chow diet (cholesterol level 0.024%) or this diet containing added cholesterol at a level of 0.12, 0.24, or 0.50% for a total of 10 days. The percent cholesterol absorption, as well as the plasma and hepatic total cholesterol concentrations in these groups of animals, are given in **Table 2.** It was found that, as the dietary cholesterol load increased, there was a fall in the percent cholesterol absorption from 49.3 ± 3.1% in the animals fed the chow diet only (Group A), to 37.1 ± 2.5% in the hamsters given chow containing added cholesterol at a level of 0.50% (Group D) ($P < 0.05$). While the overall change in the percent absorption was modest compared to the increase in the dietary cholesterol level, the amount of dietary cholesterol absorbed per day changed dramatically as the intake of cholesterol increased. The data in the last two columns of Table 2 show that the concentration of cholesterol in the plasma, and particularly in the liver, closely mirrored the differences among the various groups in the amount of dietary cholesterol that they absorbed each day.

The second manipulation examined the effect of feeding CI-976, a newly developed ACAT inhibitor (36, 38, 39), on the efficiency of cholesterol absorption. This agent was added at a level of 0.12% wt/wt (equivalent to a dose of about 110 mg/kg bw per day) to the chow diet containing 0.12% cholesterol. The data for the group fed CI-976

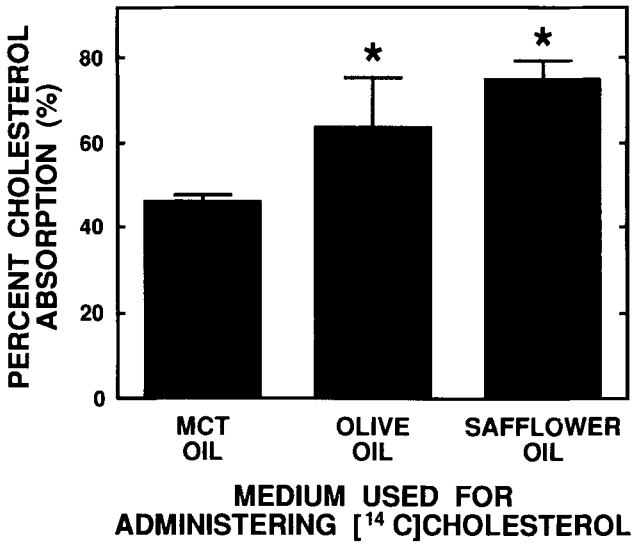

Fig. 1. Comparison of the percent cholesterol absorption in male cholesterol-fed hamsters that were dosed intragastrically with [^{14}C]cholesterol contained in either MCT oil, olive oil, or safflower oil. Male, virus-free hamsters were fed ad lib a chow diet containing added cholesterol (0.12% wt/wt) for a total of 10 days. On the seventh day all of the hamsters were given an intragastric dose of [^{14}C]cholesterol in either MCT oil, olive oil, or safflower oil, and an intravenous dose of [^{3}H]cholesterol in Intralipid. Three days later the hamsters were anesthetized with diethyl ether and bled from the abdominal aorta. Aliquots of plasma were counted to determine the content of ^{3}H and ^{14}C, and these data were used to calculate the percent cholesterol absorption according to the dual-isotope plasma ratio method. Values are the mean ± 1 SEM of data obtained from eight animals in each group. An asterisk indicates that the value is significantly different ($P < 0.05$) from the value for the group dosed using MCT oil.

TABLE 2. Percent cholesterol absorption and plasma and hepatic cholesterol concentrations in male hamsters fed a chow diet containing added cholesterol alone or cholesterol together with an ACAT inhibitor

Diet	Number of Animals	Body Weight		Liver Weight	Percent Cholesterol Absorption	Mass of Dietary Cholesterol Absorbed	Hepatic Total Cholesterol Concentration	Plasma Total Cholesterol Concentration
		Day 0	Day 10					
		g		*g*	*%*	*mg/day*	*mg/g*	*mg/dl*
A. Chow only	16	110 ± 1	126 ± 1	5.73 ± 0.15	49.3 ± 3.1	1.3	1.80 ± 0.03	123.9 ± 2.7
B. Chow + cholesterol (0.12%)	12	109 ± 1	123 ± 2	6.08 ± 0.22	43.1 ± 2.7	6.5	7.09 ± 0.51[a]	191.1 ± 7.1[a]
C. Chow + cholesterol (0.24%)	11	109 ± 1	124 ± 2	6.34 ± 0.15[a]	41.3 ± 2.9	11.4	13.59 ± 1.08[a]	220.0 ± 7.3[a]
D. Chow + cholesterol (0.50%)	12	109 ± 1	127 ± 3	6.85 ± 0.18[a]	37.1 ± 2.5[a]	20.6	23.52 ± 0.97[a]	276.3 ± 10.2[a]
E. Chow + cholesterol (0.12%) + CI-976 (0.12%)	12	111 ± 1	124 ± 2	5.16 ± 0.16[b]	22.8 ± 1.2[b]	3.5	1.98 ± 0.04[b]	131.4 ± 3.0[b]

Male, virus-free hamsters were fed a chow diet containing either added cholesterol at three different levels (0.12, 0.24, or 0.50% wt/wt) or added cholesterol (0.12%) together with CI-976, an ACAT inhibitor (0.12%). The animals were fed these diets ad lib for a total of 10 days. On the seventh day all of the hamsters were given an intragastric dose of [^{14}C]cholesterol (in MCT oil) and an intravenous dose of [^{3}H]cholesterol (in Intralipid). Three days later the hamsters were anesthetized with diethyl ether and bled from the abdominal aorta into syringes containing EDTA as anticoagulant. The liver was removed and weighed. Plasma and liver total cholesterol concentrations were measured. Aliquots of plasma were counted to determine its content of ^{3}H and ^{14}C, and these data were used to calculate the percent cholesterol absorption according to the dual-isotope plasma ratio method. The approximate mass of cholesterol absorbed each day was calculated using a total food intake of 9 g/day per 100 g body weight. The basal cholesterol level of the chow diet was 0.024%. Values are the mean ± 1 SEM of data obtained from the number of animals shown for each group.

[a] $P < 0.05$ compared to value for Group A.

[b] $P < 0.05$ compared to value for Group B.

are included in Table 2 as these animals were fed concurrently with those receiving the diets containing different levels of added cholesterol. Compared to their respective control group (Group B), the hamsters given CI-976 (Group E) had a markedly lower level of cholesterol absorption (22.8 ± 1.2% versus 43.1 ± 2.7%, $P < 0.05$). It should be noted that while the plasma levels of both [^{3}H]- and [^{14}C]cholesterol decreased in the hamsters fed the inhibitor, the proportion of the intragastric label (^{14}C) remaining in the plasma at 72 h fell 3-fold more than did the proportion of the intravenous label (^{3}H) that remained. The low level of cholesterol absorption calculated from these data for Group E was fully consistent with the finding that in these animals the plasma and hepatic cholesterol concentrations decreased to levels similar to those found in the hamsters fed the chow diet only (Group A).

The next two experiments tested the technique further by applying it to hamsters in which either the composition of the bile acid pool or the intraluminal content of lipid had been manipulated. To change the composition of the bile acid pool, the hamsters were fed the chow diet containing either cholic acid (0.10% wt/wt) or ursodeoxycholic acid (0.10%) along with added cholesterol (0.12%). While a dietary level of 0.10% of these bile acids corresponds to a dose of about 90 mg/kg bw per day, this was done to achieve a rapid and pronounced change in pool composition (18, 25, 40, 41). The level of cholesterol absorption was found to differ appreciably depending on which bile acid was fed. In the group given the chow diet containing added cholesterol, but no bile acid, the mean level of absorption was 46.8 ± 2.7%. Compared to this control group, the animals fed cholic acid had a significantly higher level of absorption (58.6 ± 2.2%, $P < 0.05$), while the opposite effect occurred in the hamsters given ursodeoxycholic acid (34.6 ± 2.5%, $P < 0.05$). These data are presented on an individual animal basis in **Fig. 2** where the hepatic total cholesterol concentration has been plotted as a function of the percent cholesterol

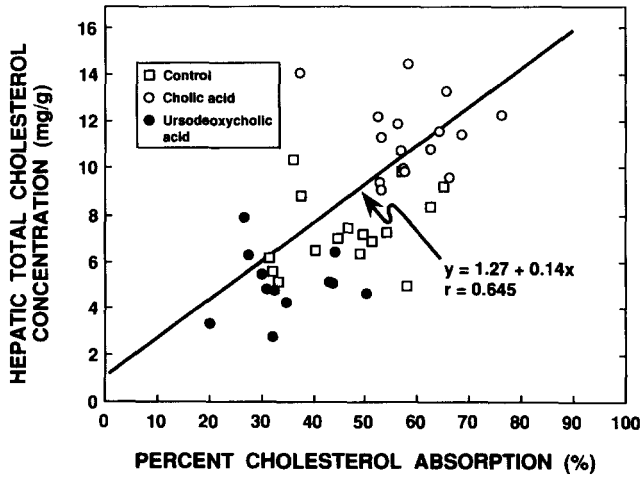

Fig. 2. Hepatic total cholesterol concentration as a function of the percent cholesterol absorption in male hamsters fed a chow diet containing added cholesterol and either ursodeoxycholic acid or cholic acid. Male, virus-free hamsters were fed ad lib a chow diet containing either added cholesterol (0.12% wt/wt) only or cholesterol (0.12%) together with either ursodeoxycholic acid (0.10%) or cholic acid (0.10%) for a total of 10 days. On the seventh day all of the hamsters were given an intragastric dose of [^{14}C]cholesterol (in MCT oil) and an intravenous dose of [^{3}H]cholesterol (in Intralipid). Three days later the hamsters were anesthetized with diethyl ether and bled from the abdominal aorta. The livers were removed, and weighed, and hepatic total cholesterol concentrations were measured. Aliquots of plasma were counted to determine the content of ^{3}H and ^{14}C, and these data were used to calculate the percent cholesterol absorption according to the dual-isotope plasma ratio method. Each point represents values from a single animal. There were 16 animals in each of the control and cholic acid-fed groups, and 12 animals in the group given ursodeoxycholic acid. The slope of the regression line is significantly different from zero ($P < 0.05$).

absorption. Overall, there was a strong positive correlation between the two variables ($r = 0.645$, $P < 0.05$). These data were thus taken as further confirmation that the values for the percent absorption obtained with this technique do faithfully reflect the mass movement of cholesterol across the intestinal wall, as it is well documented that, in cholesterol-fed animals, hepatic cholesterol levels vary directly with the amount of cholesterol absorbed from the diet (2).

In the related experiment, other groups of hamsters were fed the chow diet with either added cholesterol (0.12%) only or with cholesterol plus safflower oil at a level of 10% or 20%. Because the plain chow diet has an inherent lipid content of about only 4%, the diets with safflower oil substantially raised the amount of fat in the intraluminal contents of the small intestine. As shown in **Fig. 3**, this caused a modest but consistent increase in cholesterol absorption (panel A). Thus, in the group fed the diet without any added oil, the level of absorption was 49.6 ± 3.5%, whereas in the animals fed the diets with either 10% or 20% safflower oil, the percent absorption was 58.8 ± 4.2% ($P > 0.05$) and 61.3 ± 4.9% ($P > 0.05$),

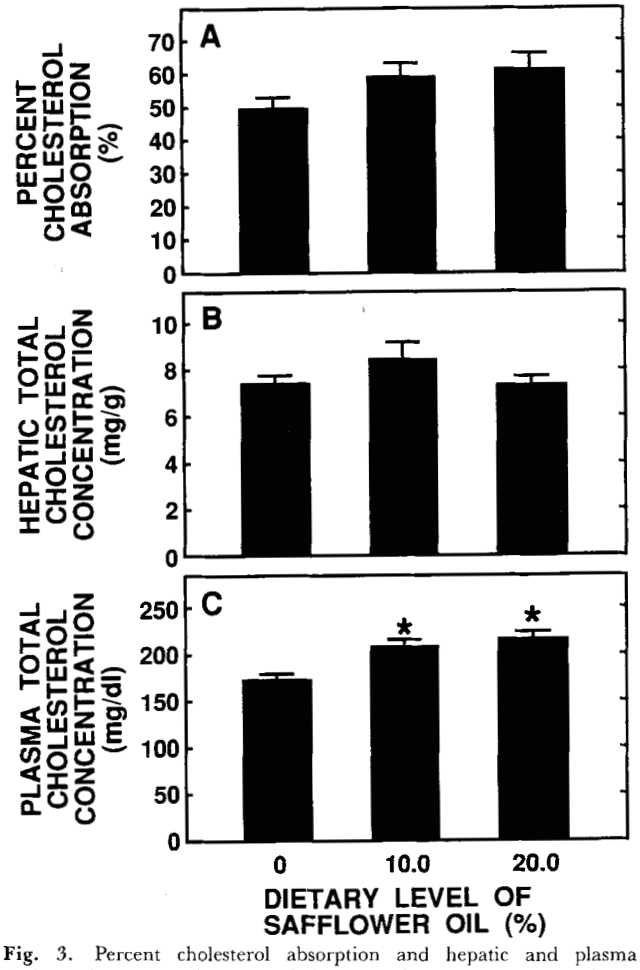

Fig. 3. Percent cholesterol absorption and hepatic and plasma cholesterol concentrations in male hamsters fed a chow diet containing added cholesterol and safflower oil. Male, virus-free hamsters were fed ad lib a chow diet containing either added cholesterol (0.12% wt/wt) only or cholesterol (0.12%) together with safflower oil at a level of either 10 or 20% (wt/wt) for a total of 10 days. On the seventh day all of the hamsters were given an intragastric dose of [^{14}C]cholesterol (in MCT oil) and an intravenous dose of [^{3}H]cholesterol (in Intralipid). Three days later the hamsters were anesthetized with diethyl ether and bled from the abdominal aorta. The livers were removed and weighed. Plasma and liver total cholesterol concentrations were measured. Aliquots of plasma were counted to determine the content of ^{3}H and ^{14}C, and these data were used to calculate the percent cholesterol absorption according to the dual-isotope plasma ratio method. Values are the mean ± 1 SEM of data obtained from eight animals in each group. An asterisk indicates that the value is significantly different ($P < 0.05$) from the values for the group not fed safflower oil.

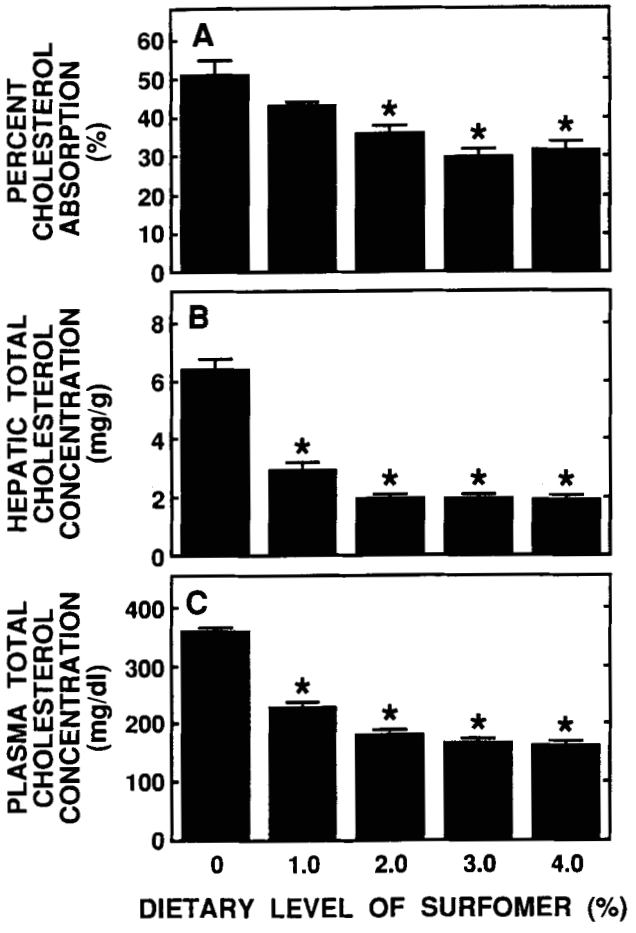

Fig. 4. Percent cholesterol absorption and hepatic and plasma cholesterol concentrations in male hamsters fed a chow diet containing added cholesterol and coconut oil and different levels of surfomer. Male, virus-free hamsters were fed ad lib a chow diet containing added cholesterol (0.12% wt/wt) and coconut oil (10% wt/wt) together with surfomer at a level of 0, 1, 2, 3, or 4% (wt/wt) for a total of 10 days. On the seventh day all of the hamsters were given an intragastric dose of [^{14}C]cholesterol (in MCT oil) and an intravenous dose of [^{3}H]cholesterol (in Intralipid). Three days later the hamsters were anesthetized with diethyl ether and bled from the abdominal aorta. The livers were removed and weighed. Plasma and liver total cholesterol concentrations were measured. Aliquots of plasma were counted to determine the content of ^{3}H and ^{14}C, and these data were used to calculate the percent cholesterol absorption according to the dual-isotope plasma ratio method. Values are the mean ± 1 SEM of data obtained from six animals in each group. An asterisk indicates that the value is significantly different ($P < 0.05$) from the value for the group not fed surfomer.

respectively. While hepatic cholesterol concentrations (panel B) did not differ significantly among the three groups, plasma cholesterol levels (panel C) were significantly elevated in the animals fed the diets with 10% and 20% safflower oil.

In the remaining two experiments, cholesterol absorption was measured in hamsters fed either surfomer or cholestyramine, both of which are synthetic, non-absorbable polymers that exert their primary cholesterol lowering action within the small intestine (34). Cholestyramine acts primarily by binding bile acids, thereby interrupting their enterohepatic circulation (42). In contrast, surfomer (AOMA), a copolymer of maleic acid and an 18-carbon α-olefin, specifically blocks the mucosal uptake of cholesterol without affecting the enterohepatic flux of bile acids (12, 43). Surfomer and cholestyramine were incorporated separately at levels of 0, 1, 2, 3, or 4% into a chow diet containing added cholesterol (0.12%) and coconut oil (10%). The results of the experiment with surfomer are given in **Fig. 4.** A clear dose-related inhibition of cholesterol absorption (panel A) was achieved, with a maximal effect being apparent at a dietary level of 3%. A significant fall in both hepatic (panel B) and plasma (panel C) cholesterol concentrations occurred at all doses, again with a maximal reduction being evident at a dietary surfomer level of 2–3%. The data for the matching study with cholestyramine are given in **Fig. 5.** With respect to the efficiency of cholesterol absorption (panel A), there was no effect at a level of 1%, but when the dietary level of the resin was raised to 4%, the percent absorption fell much more than it did with the diet containing 4% surfomer. The lower doses of cholestyramine, while having no or little effect on cholesterol absorption, essentially normalized hepatic cholesterol levels (panel B), and also significantly lowered plasma cholesterol concentrations (panel C).

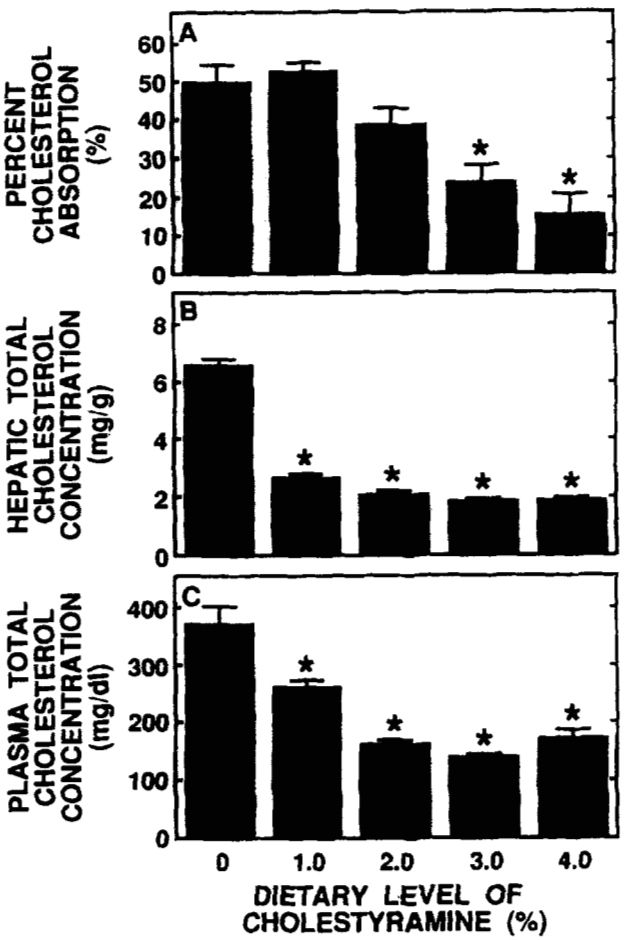

Fig. 5. Percent cholesterol absorption and hepatic and plasma cholesterol concentrations in male hamsters fed a chow diet containing added cholesterol and coconut oil and different levels of cholestyramine. Male, virus-free hamsters were fed ad lib a chow diet containing added cholesterol (0.12% wt/wt) and coconut oil (10% wt/wt) together with cholestyramine at a level of 0, 1, 2, 3, or 4% (wt/wt) for a total of 10 days. On the seventh day all of the hamsters were given an intragastric dose of [^{14}C]cholesterol (in MCT oil) and an intravenous dose of [^{3}H]cholesterol (in Intralipid). Three days later the hamsters were anesthetized with diethyl ether and bled from the abdominal aorta. The livers were removed and weighed. Plasma and liver total cholesterol concentrations were measured. Aliquots of plasma were counted to determine the content of ^{3}H and ^{14}C, and these data were used to calculate the percent cholesterol absorption according to the dual-isotope plasma ratio method. Values are the mean ± 1 SEM of data obtained from six animals in each group. An asterisk indicates that the value is significantly different ($P < 0.05$) from the value for the group not fed cholestyramine.

DISCUSSION

The present studies demonstrate how the modified dual-isotope plasma ratio method can be broadly applied to the measurement of cholesterol absorption in the hamster. Irrespective of the intended application of the technique, the values for the percent absorption that are obtained will vary greatly depending on how the method is carried out. Two particular points warrant emphasis here. First, if the hamsters are fasted before and after dosing, the percent absorption will be lower. Without appreciable amounts of food in the gastrointestinal tract, the intragastric dose of labeled cholesterol in the MCT oil obviously passes quickly through the small intestine, resulting in a significant underestimation of the level of absorption. While this problem apparently does not occur when fasted animals are given the intragastric dose in an oil phase containing large quantities of unlabeled cholesterol, bile acid, and other solubilizers, it is questionable whether this approach is the best way of measuring the effect of a particular drug or diet on the overall efficiency of intestinal sterol absorption, especially in smaller animals. It would seem important that the intragastric dose of labeled cholesterol be allowed to mix thoroughly in the stomach and proximal small bowel with the particular agent or test diet that is being evaluated. The principal concern about dosing animals in the fed state is whether they will regurgitate any of the intragastric dose during or after recovery

from anesthesia. While this was never a problem with hamsters dosed under ether anesthesia, emesis may occur in the larger animals such as the nonhuman primates after being dosed in the fed state.

The other point to emphasize about this modified protocol concerns the selection of the vehicle for administering the intragastric dose of labeled cholesterol. While the basis for the different efficiencies of cholesterol absorption that were obtained with MCT, olive, and safflower oil is not fully understood, it may be directly related to the type and proportion of unsaturated fatty acids in each oil, and to the rate at which different oils are metabolized by the intestinal cells. Unlike olive and safflower oil, which contain predominantly the unsaturated fatty acids, oleic and linoleic acid, respectively, MCT oil consists almost exclusively of two saturated fatty acids, caprylic and capric acid (29–31). MCT oil was selected as the vehicle for use in the standard dosing procedure because its constituent fatty acids have an essentially neutral effect on cholesterol metabolism (29, 31).

While the standard protocol that we adopted provides a more practical approach to the routine measurement of cholesterol absorption in the hamster, at least four assumptions are implicit in the data that are generated with this technique. First, in dosing the animals in the fed state, it is assumed that they all have a significant quantity of food in their stomachs. This is a reasonable assumption in the case of the Golden Syrian hamster because, when fed ad lib, this species not only consumes multiple meals during the day, but these meals are equally spaced throughout each 24-h period (44). Clearly, in other species like the rat, which has a pronounced diurnal rhythm in its feeding pattern, the optimum time for dosing the animals would be at about the mid-point of the dark period when food consumption is greatest. Second, it is assumed that all of the [^{14}C]- and [^{3}H]cholesterol in the plasma reflects only the net proportion of the original dose of each radiolabel that was given 72 h earlier. Since hamsters practice coprophagy, this could potentially result in an overestimation of the percent cholesterol absorption to the extent that there is any recirculation of unmetabolized labeled cholesterol from the feces back into the plasma. However, as has been reported for the rat (9), the level of cholesterol absorption in hamsters was essentially the same irrespective of whether they had free access to their stools after dosing. The third assumption is that the level of [^{14}C]cholesterol in the plasma after 72 h is reflective of the efficiency with which not only dietary, but also biliary and other endogenously derived cholesterol, is absorbed. While it is very difficult to show that this is in fact the case, it would seem more likely to be so in animals that are fed before and after dosing than in those that are subjected to an extensive period of fasting. The fourth assumption is common to almost all techniques for measuring cholesterol absorption, and concerns the unknown extent to which the labeled cholesterol given intragastrically exchanges with unlabeled cholesterol within the membranes of the intestinal cells (8). While there is some evidence that this may be a minimal source of error in cholesterol absorption measurements in humans (45), it remains unclear whether this might also be the case in experimental animals.

Together, these various points underscore the difficulty in obtaining a quantitative measure of cholesterol absorption in animal models like the hamster. Nevertheless, the application of this modified plasma ratio method to hamsters subjected to an array of experimental manipulations has yielded significant new and useful information concerning the physiology and pharmacologic regulation of intestinal cholesterol absorption in this species. With respect to the characteristics of absorption in the adult male hamster maintained on a cholesterol-enriched chow diet, several points can now be made. First, as illustrated in **Fig. 6,** the values for the percent absorption in control hamsters, drawn from a combination of experiments, fall in almost exactly the same range as the values that have been reported for normal humans (43, 46, 47). Second, in the weight range in which the hamster is normally used for metabolic studies (i.e., 110–160 g), the percent cholesterol absorption remains remarkably constant. Third, the percent absorption falls as the level of cholesterol in the diet is increased. The same effect has been described for the African green monkey and baboon and, occasionally but not always, for humans (3, 7, 46–49). Fourth, the efficiency of cholesterol absorption is modestly increased as the total lipid content of the diet is raised.

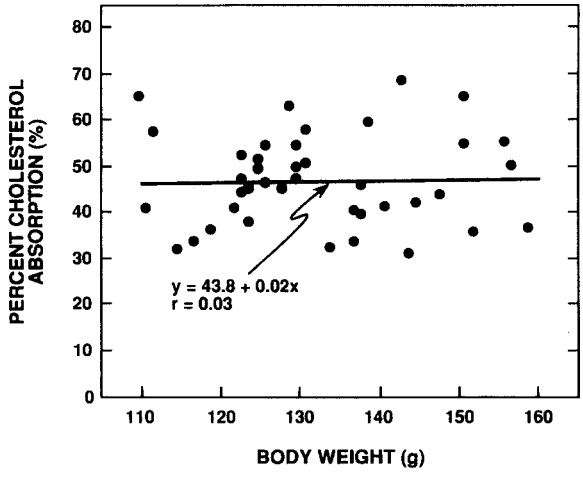

Fig. 6. Percent cholesterol absorption as a function of body weight in male hamsters fed a chow diet containing a fixed level of added cholesterol. Male, virus-free, hamsters were fed ad lib a chow diet containing added cholesterol (0.12% wt/wt) for a total of 10 days. The percent cholesterol absorption in each hamster was measured as described in the preceding figure legends. These values represent the data for 40 individual animals that served as the controls for the experiments described in Tables 1 and 2, and Figs. 1, 2, and 3. The slope of the regression line is not significantly different from zero ($P > 0.05$).

Presumably this occurs largely because the increased availability of fatty acids in the intestinal lumen promotes the micellerization and uptake of cholesterol by the mucosal cells. Fifth, the percent cholesterol absorption shifts significantly depending on the composition of the bile acid pool. The fall in the efficiency of absorption with ursodeoxycholic acid feeding is well documented in humans, and has also been reported previously for the hamster (18, 50, 51). The increase in absorption seen in the hamsters given cholic acid is consistent with the effect described in the rat (52). Sixth, except in the case of the hamsters fed cholestyramine, hepatic cholesterol concentrations generally vary directly with the efficiency of cholesterol absorption.

The particular pharmacologic manipulations that were applied to the hamsters in these studies were selected because of their established effects on cholesterol absorption in humans, and, to a limited extent, in other animal models. Although only a single dose of the new ACAT inhibitor CI-976 was tested, it dramatically inhibited absorption, normalized hepatic cholesterol content, and reversed the hypercholesterolemia that developed in the cholesterol-fed hamsters. It should be emphasized that the precision of the cholesterol absorption measurements made in the hamster given CI-976 was probably unaffected by any action that this inhibitor may have exerted directly on hepatic ACAT activity and biliary cholesterol secretion. The finding that at 72 h the proportion of the [^{14}C]cholesterol dose remaining in the plasma fell markedly while there was little change in the fraction of the [^{3}H]cholesterol dose that remained, showed that CI-976 acted principally at the level of the intestine. The halving of the percent cholesterol absorption by this agent fitted well with its strong hypocholesterolemic action.

The dose-related inhibition of absorption induced by surfomer was evident at a dietary level of only 1%. At higher doses, the accumulation of any excess cholesterol in the liver was prevented and plasma cholesterol levels fell significantly. Cholestyramine, at a dietary level of only 1%, dramatically lowered hepatic and plasma cholesterol concentrations, but caused no measurable change in absorption. Clearly, at this dose, the liver was able to fully compensate for the resin-induced loss of bile acid, thereby preventing any reduction in cholesterol absorption, while simultaneously diminishing both plasma and hepatic cholesterol concentrations. However, at dietary levels of 2% or more, the proportion of the bile acid pool that was either lost or bound was sufficiently great to dramatically reduce the efficiency of cholesterol absorption. This was accompanied by a further lowering of plasma and hepatic cholesterol levels. Thus, at these higher doses, the hypocholesterolemic action of cholestyramine in the hamster is mediated through two interrelated mechanisms.

Two points about these dose response data for surfomer and cholestyramine are noteworthy. One is that they will be very useful as reference values in future studies investigating the efficacy and mechanism of action of new cholesterol-lowering agents in the hamster and similar animal models. The other point is that the data for the low doses of surfomer reveal how relatively small reductions in the percent of cholesterol absorption are accompanied by much more dramatic decreases in the level of cholesterol in the liver and plasma. Such data imply that the wide individual differences in dietary cholesterolemic response found in many species including humans may indeed be due partly to relatively small inherent differences between those individuals in the efficiency with which they absorb dietary cholesterol. If this is the case, then the development of more effective inhibitors of cholesterol absorption should provide an attractive alternative for regulating plasma cholesterol levels in the general population.

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