methods

Measurement of intestinal cholesterol absorption by plasma and fecal dual-isotope ratio, mass balance, and lymph fistula methods in the mouse: an analysis of direct versus indirect methodologies

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Abstract The rate of intestinal cholesterol (Ch) absorption is an important criterion for quantitation of Ch homeostasis. However, studies in the literature suggest that percent Ch absorption, measured usually by a fecal dual-isotope ratio method, spans a wide range, from 20% to 90%, in healthy inbred mice on a chow diet. In the present study, we adapted four standard methods, one direct (lymph collection) and three indirect (plasma and fecal dual-isotope ratio, and sterol balance) measurements of Ch absorption and applied them to mice. Our data establish that all methodologies can be valid in mice, with all methods supporting the concept that gallstone-susceptible C57L mice absorb significantly more Ch (37 ± 5%) than gallstone-resistant AKR mice (24 ± 4%). We ascertained that sources of error in the literature leading to marked differences in Ch absorption efficiencies between laboratories relate to a number of technical factors, most notably expertise in mouse surgery, complete solubilization and delivery of radioisotopes, appropriate collection periods for plasma and fecal samples, and total extraction of radioisotopes from feces. We find that all methods provide excellent interexperimental agreement, and the ranges obtained challenge previously held beliefs regarding the spread of intestinal Ch absorption efficiencies in mice. The approaches documented herein provide quantifiable methodologies for exploring genetic mechanisms of Ch absorption, and for investigating the assembly and secretion of chylomicrons, as well as intestinal lipoprotein metabolism in mice.—Wang, D. Q-H., and M. C. Carey. **Measurement of intestinal cholesterol absorption by plasma and fecal dual-isotope ratio, mass balance, and lymph fistula methods in the mouse: an analysis of direct versus indirect methodologies.** ***J. Lipid Res.*** **2003.** 44: **1042–1059.**

Supplementary key words nutrition • genetics • lecithin • bile salts • bile flow • chylomicrons • sitostanol • radiolabeled sterols

The small intestine is the organ uniquely responsible for partial absorption of both dietary and biliary cholesterol (Ch), and plays a major role in regulation of whole-body Ch balance (1–3). Accurate and precise measurements of intestinal Ch absorption is one of the basic requirements for quantitation of Ch homeostasis as well as for ascertaining genetic variations and drug effects. Presently, there are three indirect methods available for measuring Ch absorption. Of these, the simplest is the plasma dual-isotope ratio method introduced by Zilversmit (4), recently modified by us for the mouse (5, 6). The method is based on the simultaneous ig and iv administration of [^{3}H]Ch and [^{14}C]Ch and measurement of plasma Ch isotope ratios at a set point in time: by definition, it assumes "100% absorption" of the iv dose. The most frequently employed method in the literature is the fecal dual-isotope ratio method (7), which is based on the administration of a single oral dose of radiolabled Ch and a nonabsorbed radiolabled phytosterol, such as β-sitosterol (7, 8) or sitostanol (9), as reference marker. The measurement is critically dependent on an accurate assessment of the ratios of excreted radioactivities of the two isotopes and their bacterial metabolites in feces. In a metabolic steady state, the sterol balance method (10–12) estimates the mass absorption of exogenous Ch based on the difference between dietary Ch and its fecal excretion. The measurement of endogenous fecal neutral steroid excretion is the key to the validity of these calculations, since there are significant variations from 5 μmol/h/kg to 10 μmol/h/kg in biliary Ch outputs in mice (6). Obviously, the direct measurement of intestinal Ch absorption is to determine the lymphatic transport of a radioisotope (13–15). All of these

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Abbreviations: ABC, ATP binding cassette; Ch, cholesterol.

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TABLE 1. Species comparison on efficiency of intestinal cholesterol absorption

Species	Method[a]	Ch Absorption[b]	Reference
		%	
Rat	Lymph	40–45	(14, 16, 17)
	Balance	—[c]	
	Plasma	38–55	(4, 18)
	Fecal	55–60	(7, 19)
Hamster	Lymph	—	
	Balance	67	(20)
	Plasma	40–58	(9, 21)
	Fecal	44–50	(9)
Guinea pig	Lymph	—	
	Balance	—	
	Plasma	30–47	(22)
	Fecal	34–55	(22)
Rabbit	Lymph	60–80	(23, 24)
	Balance	—	
	Plasma	Unstable[d]	(25)
	Fecal	71–85	(26, 27)
Dog	Lymph	80	(28, 29)
	Balance	81	(30)
	Plasma	70–76	(30)
	Fecal	70–89	(28, 30)
Primate	Lymph	34–65	(31, 32)
	Balance	37–55	(33–35)
	Plasma	43–46	(35, 36)
	Fecal	34–54	(34–38)
Human	Lymph	31–45[e]	(39, 40)
	Balance	38–48	(10, 41–43)
	Plasma	42–52	(41, 44–47)
	Fecal	40–55	(44, 45, 48)

Ch, cholesterol.

[a] For sake of uniformity, the data cited are limited to those using one of the four methods that were studied in the present paper. A "zero" Ch or low Ch diet was fed during these Ch absorption studies.

[b] The values represent the range of percent Ch absorption reported in the literature.

[c] No information on percent Ch absorption data available in the literature.

[d] Percent Ch absorption is overestimated and variable in rabbits by this method, due to the fact that rabbits fail to release the iv dose into plasma even over an extended period. See Discussion for further description.

[e] All subjects were clinically healthy at the time of study, and did not suffer from obesity or hyperlipidemia.

methods have been validated and used extensively in rats (4, 7, 13, 14, 16–19), hamsters (9, 20, 21), guinea pigs (22), rabbits (23–27), dogs (28–30), and primates (31–38), including humans (10, 39–48) (**Table 1**).

However, there are contradictory results in the literature concerning the percent of Ch absorption in healthy inbred mice fed chow, reported to vary from 20% to 90%. This extraordinary range for interstrain variation was determined mostly by the fecal dual-isotope ratio method (6, 49–75) (**Table 2**). Few studies were carried out in mice using the plasma dual-isotope ratio method (5, 6, 75–79) (**Table 3**). These divergent findings suggest that methods for measurement of Ch absorption in mice should be reevaluated. In particular, the availability of a large number of inbred strains, including congenic and recombinant inbred mice and the well-defined homology between the human and mouse genomes (79), all make the inbred mouse a surrogate species for the identification of genes regulating intestinal Ch absorption in humans. Furthermore, the mouse is an important model for the study of complex genetic traits, and its use in genetic studies reduces the interference of environmental differences and the problems incurred by genetic heterogeneity (80, 81).

In the present study, we used the higher Ch-absorbing C57L mice and the lower Ch-absorbing AKR mice (6) to *i*) evaluate and compare fecal, plasma, and lymphatic dual isotopes, as well as sterol balance methods for measuring intestinal Ch absorption; *ii*) develop, validate, and establish standard methods for studying mouse Ch absorption; and *iii*) investigate how dietary and biliary Ch levels and bile acid feeding influence intestinal Ch absorption. This study, and the modified methods documented herein, should provide a basic framework for investigating genetic determinants that regulate intestinal Ch absorption in the mouse.

MATERIALS AND METHODS

Chemicals

Intralipid (20%, wt/v) was purchased from Pharmacia (Clayton, NC), and medium-chain triglyceride (MCT) was from Mead Johnson (Evansville, IN). Ch oxidase and Ch esterase were obtained from Sigma (St. Louis, MO). Corn oil was purchased from Catania-Spagnae (Ayer, MA), culinary olive oil was from the Pastene Companies (Canton, MA), sunflower oil was from Shaw's Supermarkets (East Bridgewater, MA), safflower oil was from the Hain Food Group (Uniondale, NY), peanut oil was from Nabisco (East Hanover, NJ), and canola (rapeseed) oil, vegetable (soybean) oil, and skim milk were from Star Markets (Cambridge, MA). The radioisotopes [1,2-^{3}H]Ch, [4-^{14}C]Ch, and [9,10-^{3}H(N)]palmitic acid were purchased from NEN Life Science Products (Boston, MA), and [5,6-^{3}H]sitostanol was from American Radiolabeled Chemicals (St. Louis, MO).

Animals and diets

Male C57L/J and AKR/J inbred mice, 6–8 wk old, were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were maintained in a temperature-controlled room (22 ± 1°C) with a 12 h light cycle (6 AM–6 PM). Throughout the experimental period, mice were provided free access to water and Purina laboratory chow (Mouse Diet 1401, St. Louis, MO) containing trace Ch (<0.02%) (5, 82, 83), and were allowed to adapt to the environment for 2 weeks prior to the Ch absorption study. To curtail coprophagy during the study, animals were housed in individual cages with wire mesh bottoms (5, 6). All experiments were executed according to accepted criteria for the care and experimental use of laboratory animals, and euthanasia was consistent with recommendations of the American Veterinary Medical Association. All protocols were approved by the Institutional Animal Care and Use Committee of Harvard University.

Measurement of Ch absorption via lymphatic Ch transport

Male AKR and C57L mice (n = 5 per group) were fasted overnight but allowed free access to water. Animals were weighed and anesthetized by an ip injection of pentobarbital (Abbott Laboratories, North Chicago, IL) at a dose of 35 mg/kg. Laparotomy was performed under sterile conditions through an upper-midline incision. For better visualization of the mesenteric lymphatic duct (84, 85), each animal was dorsally arched over a 3 ml syringe (OD 12 mm). With magnification provided by a zoom stereomicroscope (Olympus America, Melville, NY), a PE-10 polyethylene catheter was inserted into the mesenteric lymphatic

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TABLE 2. Percent Ch absorption determined by a fecal dual-isotope ratio method in chow-fed mice: an inventory of results reported in the literature

Mouse	Gender	n	Dose	Vehicle	Nonabsorbed Marker	Fecal Collection	Ch Absorption	Reference
			μl			*days*	*%*	
Inbred strains								
AKR/J	M	10	150	MCT oil	I[a]	4	29 ± 5	(6)
	F	6	50	Lipid emulsion[b]	II	1	72 ± 5	(49)
	M	8–10	100	MCT oil	I	3	63 ± 2	(50, 51)
BALB/cJ	M	10	150	MCT oil	I	4	33 ± 5	(6)
	F	6	50	Lipid emulsion	II	1	71 ± 8	(49)
	F	5	—[c]	Chow+olive oil	II	—	31 ± 3	(52)
C3H/J	M	10	150	MCT oil	I	4	28 ± 4	(6)
C3H/HeJ	F	5	SF[d]	Chow+safflower oil	II	3	84 ± 4	(53)
	F	5	SF	Chow	II	3	79 ± 3	(53)
	F	6	50	Lipid emulsion	II	1	72 ± 3	(49)
	F	5	SF	Chow+butter oil	II	3	70 ± 3	(53)
C57BL/6ByJ	F	6	100	Safflower oil	I	4	59 ± 4	(54)
C57BL/6J	M	8-10	100	MCT oil	I	3	42 ± 4	(50, 51)
	M	5	100	Corn oil	I	1	80 ± 7	(55)
	M	5	100	Olive oil	I	1	76 ± 10	(56)
	M	5	100	Safflower oil	I	5	50 ± 19	(57)
	M	4	—	MCT oil	I	1	39 ± 4	(58)
	M	9	—	MCT oil	I	3	20 ± 3	(58)
	F	5	SF	Chow+safflower oil	II	3	81 ± 1	(53)
	F	5	50	PL vesicles	I	1	78 ± 6	(59)
	F	5	50	Lipid emulsion	I	1	76 ± 4	(59)
	F	6	50	Lipid emulsion	II	1	75 ± 5	(49)
	F	5	SF	Chow	II	3	74 ± 4	(53)
	F	5	SF	Chow+butter oil	II	3	70 ± 4	(53)
	F	6	100	Safflower oil	I	4	60 ± 5	(54)
	F	5	—	Chow+olive oil	II	—	39 ± 5	(52)
	M+F	19	50	Lipid emulsion	I	1	66 ± 2	(60)
	M+F	3	50	PL vesicles	I	1	59 ± 2	(60)
	—	6	150	Liquid diet	I	1	78 ± 8	(61)
	—	3	100	Sunflower oil	II	4	57 ± 3	(62)
C57L/J	M	10	150	MCT oil	I	4	42 ± 7	(6)
	F	6	50	Lipid emulsion	II	1	65 ± 4	(49)
DBA/2J	M	8–10	100	MCT oil	I	3	32 ± 4	(50, 51)
	M	8	100	MCT oil	I	1	42 ± 5	(50)
	F	5	SF	Chow	II	3	74 ± 4	(53)
	F	6	50	Lipid emulsion	II	1	73 ± 5	(49)
	F	5	SF	Chow+safflower oil	II	3	57 ± 3	(53)
	F	5	SF	Chow+butter oil	II	3	56 ± 3	(53)
FVB/J	M	10	150	MCT oil	I	4	39 ± 8	(6)
SJL/J	M	8–10	100	MCT oil	I	3	28 ± 4	(50, 51)
SWR/J	M	10	150	MCT oil	I	4	32 ± 5	(6)
129P3/J	M	8–10	100	MCT oil	I	3	67 ± 2	(50, 51)
129/SvEv	M	5	—	MCT oil	I	1	63 ± 9	(58)
	M	9	—	MCT oil	I	3	44 ± 3	(58)
	F	5	SF	Chow	II	3	87 ± 4	(53)
	F	5	SF	Chow+safflower oil	II	3	83 ± 5	(53)
	F	5	SF	Chow+butter oil	II	3	81 ± 5	(53)
	F	6	50	Lipid emulsion	II	1	68 ± 1	(49)
129/J	M	10	—	MCT oil	I	3	52 ± 4	(58)
Outbred strains								
CD1	M	5	200	Gum arabic[e]	III	3	26 ± 4	(62)
	M	5	200	Gum arabic	III	3	25 ± 3	(63)
	M	13	—	MCT oil	I	3	25 ± 3	(64)
	F	15	—	MCT oil	I	3	41 ± 5	(64)
Swiss	M	4	SF	Chow+ethanol	II	1	61 ± 1	(65)
	F	6	50	Lipid emulsion	II	1	71 ± 8	(49)
	F	—	—	Albumin oleate	III	3	67	(66)

duct according to published methods (6). The catheter was then externalized through the right abdominal wall and connected with a heparinized microtube for collecting lymph by gravity. Immediately, a PE-10 catheter was inserted into the duodenum 5 mm distal to the pylorus, and secured with 6-0 silk sutures and adhesive. The duodenal catheter was externalized through the left abdominal wall and connected to an infusion pump (Kent Scientific, Litchfield, CT). Following completion of all surgical procedures, the abdomen was closed with continuous 5-0 silk sutures. A PE-10 catheter was then inserted into the jugular vein and connected to a second infusion pump. For maintenance of hydration, 0.9% NaCl was infused by iv at 100 μl/h throughout the experimental period. During surgery and lymph collection, mouse body temperature was maintained at 37°C ± 0.5°C

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TABLE 2. (*Continued*)

Mouse	Gender	n	Dose	Vehicle	Nonabsorbed Marker	Fecal Collection	Ch Absorption	Reference
			μl			*days*	%	
Mixed genetic background								
(Black Swissx129)F_2	F	—	50	Lipid emulsion	II	1	57 → 87	(49)
C57BL/6JxDBA/1LacJ	—	6	150	Liquid diet	I	1	69 ± 6	(61)
C57BL/6Jx129/SvEv	M	10	—	MCT oil	I	3	38 ± 4	(67)
	M	4	—	MCT oil	I	3	37 ± 4	(68)
	F	10	—	MCT oil	I	3	52 ± 5	(67)
	—	3	—	—	I	3	82 ± 9	(69)
	—	3	—	Safflower oil	I	7	79 ± 16	(70)
	—	5–7	—	MCT oil	I	4	44 ± 3	(71)
	—	9	—	Plant oil	I	3	84 ± 3	(72)
	M	15	100	MCT oil	I	3	55	(73)
C57BL/6Jx129S3/svlmJ	—	6	100	Corn oil	II	3	56 ± 3	(74)
C57BL/6JxBALB/cByJ	M	10	150	MCT oil	I	4	41 ± 7	(75)
Knockout strains								
ABCA1	—	3	150	Liquid diet	I	1	81 ± 9	(61)
	—	10	—	Plant oil	I	3	74 ± 8	(72)
ACAT1	—	3	—	—	I	3	74 ± 15	(69)
ApoA-I	—	6	150	Liquid diet	I	1	73 ± 6	(61)
ApoA-IV	M	5	100	Safflower oil	I	5	57 ± 16	(57)
ApoB-48	—	2	—	Safflower oil	I	7	0	(70)
ApoE	M	5	100	Olive oil	I	1	65 ± 7	(56)
	—	5–7	—	MCT oil	I	4	45 ± 3	(71)
Ch esterase	M+F	21	50	Lipid emulsion	II	1	70 ± 2	(60)
	M+F	3	50	PL vesicles	II	1	50 ± 2	(60)
Ch 7α-hydroxylase	M	4	—	MCT oil	I	3	<1	(68)
Oxysterol 7α-hydroxylase	M	10	—	MCT oil	I	3	38 ± 3	(67)
	F	10	—	MCT oil	I	3	51 ± 4	(67)
SR-B1	M	15	100	MCT oil	I	3	66	(73)
	—	10	100	Corn oil	II	3	59 ± 4	(74)
	M	10	150	MCT oil	I	4	38 ± 6	(75)

ABC, ATP binding cassette; apo, apolipoprotein; MCT, medium-chain triglyceride; PL, phospholipid; SF, solid food, i.e., chow; and SR-BI, scavenger receptor class B type I.

[a] Nonabsorbed marker: I, sitostanol; II, sitosterol; III, no marker used.

[b] Lipid emulsion containing 2 g/l Ch, 8 g/l egg phosphatidylcholine, and 50 g/l triolein.

[c] No information available in the literature.

[d] Radioisotopes were added to SF, i.e., chow or chow mixed with oils, and a total amount of 4 g of food was fed.

[e] Five percent gum arabic solution containing 20% ethanol.

with a heating lamp and monitored with a thermometer. Continuous anesthesia (83) was maintained with ip injections of pentobarbital at a dose of 17 mg/kg every 2 h. Upon written request, detailed protocols for measurement of intestinal Ch absorption by the lymphatic transport method, as well as sterol balance, and plasma and fecal dual-isotope ratio methods are available from the corresponding author.

During the recovery period, the animals were infused intraduodenally with 0.9% NaCl at 200 μl/h for 2 h. Through the duodenal catheter, we instilled 100 μl MCT containing 2.5 μCi of [^{14}C]Ch, 5 μCi [^{3}H]palmitic acid, and 0.5% taurocholate (TC). The study of absorption and lymphatic transport of Ch commenced from this time point on. To maintain steady-state intestinal lymph flow, a continuous intraduodenal infusion of MCT with 0.5% TC at 300 μl/h was given via an infusion pump. Fresh lymph was collected every hour into a heparinized microtube for a total of 12 h. To determine the proportions of [^{14}C]Ch and [^{3}H]palmitic acid that were transported in lymph, 50 μl lymph aliquots and the original dosing mixture were added to 10 ml of EcoLite (ICN Biomedicals, Costa Mesa, CA), respectively. The vials were shaken vigorously for 10 min and counted in a liquid scintillation spectrometer (Beckman Instruments, San Ramon, CA). Because cumulative radioactivities in lymph reached steady states in both strains of mice at 12 h, these radioactivities were taken as the efficacy of intestinal Ch absorption.

Measurement of Ch absorption by the sterol balance method

Additional groups of AKR and C57L mice (n = 5 per group) housed in individual metabolic cages with wire mesh bottoms were allowed to adapt to the environment for 2 weeks. When body weight, food ingestion, and fecal excretion were constant, i.e., an apparent metabolic steady-state, food intake was measured and feces were collected daily for a continuous 4 day period. Animals were then weighed and anesthetized with pentobarbital. After cholecystectomy, the common bile duct was cannulated with a PE-10 catheter, and hepatic bile was collected for the first hour (83). Bile Ch and Ch content in chow were measured by HPLC (5). Fecal neutral sterols were isolated, saponified, and extracted essentially according to the methods of Miettinen, Ahrens, and Grundy (11, 12), slightly modified by us recently for the mouse (6). The dried sterols were dissolved in 50 μl isopropanol and mixed with 250 μl PBS. After vortex mixing, a 50 μl aliquot of an enzyme reaction mixture (86) was added. The mixture contained 150 mM sodium phosphate (pH 7.0), 0.1 unit of Ch oxidase, 0.1 unit of Ch esterase, and 30 mM TC. The sterols and enzyme reaction mixture were shaken for 20 min and incubated for 1 h at 37°C. After addition of 300 μl methanol, followed by 3 ml petroleum ether, the samples were shaken for 10 min and centrifuged at 10,000 *g* for 3 min to separate the phases. A 2 ml aliquot of the upper phase was transferred to a clean tube and dried un-

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TABLE 3. Percent Ch absorption determined by a plasma dual-isotope ratio method in chow-fed mice: an inventory of results reported in the literature

Mouse	Gender	n	Dose	iv Administration Vehicle	Dose	ig Administration Vehicle	Plasma Collection Day	Ch Absorption	Reference
								%	
Inbred strains									
A/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	24 ± 5	(6)
AKR/J	M	20	100 μl	Intralipid	150 μl	MCT oil	3	24 ± 4	(6)
BALB/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	27 ± 4	(6)
C3H/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	22 ± 5	(6)
C57BL/6J	—[a]	5	—	2.5% ethanol+0.9% NaCl	—	Skim milk	2–3	68 ± 4	(76)
	—	6	—	0.9% NaCl	—	Skim milk	1	60 ± 13	(77)
	—	6	—	0.9% NaCl	—	Skim milk	2	64 ± 14	(77)
	—	6	—	0.9% NaCl	—	Skim milk	3	60 ± 14	(77)
	M	20	100 μl	Intralipid	150 μl	MCT oil	3	39 ± 5	(6)
C57L/J	M	5	100 μl	Intralipid	150 μl	MCT oil	3	34 ± 7	(5)
	M	35	100 μl	Intralipid	150 μl	MCT oil	3	34 ± 7	(6)
DBA/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	27 ± 3	(6)
FVB/NJ	—	—	—	Lipofundin S	—	MCT oil	2	70 ± 13	(78)
SJL/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	26 ± 3	(6)
SM/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	29 ± 4	(6)
SWR/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	28 ± 4	(6)
Outbred strains									
CD1	M	5	200 μl	Gum arabic[b]	100 μl	0.9% NaCl+5% ethanol	2.5	27 ± 3	(63)
	M	5	200 μl	Gum arabic	100 μl	0.9% NaCl+5% ethanol	3	20 ± 3	(62)
Mixed genetic background									
C57BL/6JxBALB/cJ	—	5	—	0.9% NaCl	—	Skim milk	3[c]	84	(79)
C57BL/6JxBALB/cByJ	M	10	100 μl	Intralipid	150 μl	MCT oil	3	36 ± 5	(75)
AKR/JxC57L/J	M	10	100 μl	Intralipid	150 μl	MCT oil	3	31 ± 5	(6)
Knockout strains									
SR-B1	M	10	100 μl	Intralipid	150 μl	MCT oil	3	32 ± 5	(75)
ApoB	—	8	—	0.9% NaCl	—	Skim milk	1	61 ± 7	(77)
	—	8	—	0.9% NaCl	—	Skim milk	2	64 ± 10	(77)
	—	8	—	0.9% NaCl	—	Skim milk	3	62 ± 7	(77)
Carboxyl ester lipase	M	9	—	0.9% NaCl	—	Skim milk	3[c]	78 ± 12	(79)
	F	6	—	0.9% NaCl	—	Skim milk	3[c]	94 ± 20	(79)
Mdr2	—	—	—	Lipofundin S	—	MCT oil	2	42 ± 8	(78)
Transgenic strains									
ApoA-IV	—	5	—	2.5% ethanol+0.9% NaCl	—	Skim milk	2–3	69 ± 5	(76)

Mdr2, multidrug resistance gene 2.
[a] Dashes imply no information available in the literature.
[b] Five percent gum arabic solution containing 20% ethanol.
[c] Percent Ch absorption was not significantly different between Days 1, 2, and 3 of the plasma sampling.

der nitrogen. The dried sterols were dissolved in 200 μl acetonitrile. Chromatography was performed with a Beckman HPLC system (Beckman Coulter, Fullerton, CA) at room temperature (22 ± 1°C) employing a mixture of methanol and acetonitrile (1:1, v/v); flow rate, 1 ml/min; detector setting, 240 nm; Ultrasphere ODS column, 5 μm, 4.6 mm × 250 mm. Calculation of percent Ch absorption was made as follows:

$$\% \text{ Ch absorption} = [(\text{daily dietary Ch intake} + \text{daily biliary Ch output} - \text{daily total fecal neutral sterol output}) \div \text{daily dietary Ch intake}] \times 100 \quad (Eq. 1)$$

Measurement of Ch absorption by the fecal dual-isotope ratio method

We investigated the relationships between food intake, fecal output, and fecal excretion of [^{14}C]Ch and [^{3}H]sitostanol after a single oral dose of the radioisotopes administered by gavage. Food intake in both AKR and C57L mice (n = 10 per group) was measured and feces collected every 24 h for 10 day periods. After drying the feces, the radioactive isotopes were saponified, extracted, and counted according to the methods described by Miettinen, Ahrens, and Grundy (11, 12), Borgström (87), and Turley, Daggy, and Dietschy (9).

To determine the percent of Ch absorption in AKR and C57L mice (n = 20 per group) by the fecal isotope ratio method (6, 75), we employed [^{3}H]sitostanol as the reference compound because we estimated in earlier studies that this saturated sterol is poorly absorbed (<3%) in mice, as indicated by the fecal recovery method (6). Nonfasted and nonanesthetized animals were given, by ig gavage, 150 μl of MCT containing 1 μCi of [^{14}C]Ch and 2 μCi of [^{3}H]sitostanol. The radioactive isotopes from 4 day pooled fecal samples were saponified, extracted, and counted. The ratio of the two radiolabels in the fecal extracts and the dosing mixture was used for calculation of percent Ch absorption:

$$\% \text{ Ch absorption} = \left(\frac{[^{14}\text{C}]/[^{3}\text{H}] \text{ dosing mixture} - [^{14}\text{C}]/[^{3}\text{H}] \text{ feces}}{[^{14}\text{C}]/[^{3}\text{H}] \text{ dosing mixture}}\right) \times 100 \quad (Eq. 2)$$

To investigate whether different time periods of combining fecal samples influence the derived Ch absorption level, we analyzed 1 day to 10 day pooled samples from AKR and C57L mice (n = 10 per group). Further, to study whether the percent Ch absorption was influenced by the type of vehicle used, we gave C57L mice (n = 10 per group) by ig gavage a mixture of 1 μCi of [^{14}C]Ch

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and 2 μCi of [^{3}H]sitostanol in 150 μl of corn, MCT, olive, peanut, rapeseed, safflower, soybean, and sunflower oils, or skim milk. The 4 day pooled fecal samples were used for calculating percent Ch absorption as described above.

Measurement of Ch absorption by the plasma dual-isotope ratio method

In other groups of AKR and C57L mice (n = 20 per group), Ch absorption was determined by the plasma dual-isotope ratio method as described previously (5, 6, 75). The ratio of two radiolabels in plasma, 3 days after dosing, was used for calculating percent Ch absorption:

$$\% \text{ Ch absorption } = \left(\frac{\text{Percent of IG dose } [^{14}\text{C}]\text{Ch per ml plasma}}{\text{Percent of IV dose } [^{3}\text{H}]\text{Ch per ml plasma}}\right) \times 100 \qquad \textit{(Eq. 3)}$$

To document the temporal changes in plasma radioactivities in AKR and C57L mice (n = 5 per group at each time point), plasma samples were obtained and the radioactivities were counted at 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 98 h, and 120 h after the iv injection of [^{3}H]Ch and ig administration of [^{14}C]Ch.

Cannulation of the common bile duct and collection of hepatic bile

To examine the effects of dietary Ch and bile acid feeding on Ch absorption efficiency, C57L mice (n = 10 per group) were fed chow (<0.02% Ch), a lithogenic diet containing 1% Ch and 0.5% cholic acid (CA), or semisynthetic diet containing 0.5%, 1%, or 2% Ch, 0.5% ursodeoxycholic acid (UDCA), or 0.5% CA for 1 week. The same animals were used for studies of both biliary lipid secretion (83) and intestinal Ch absorption by the plasma dual-isotope ratio method (5, 6, 75). Total and individual bile salt concentrations, as well as bile Ch and Ch content in chow, were determined by HPLC (5, 6, 82, 83). Hydrophobicity indices of hepatic biles were calculated according to Heuman's method (88).

Statistical analyses

All data are expressed as means ± SD. Statistically significant differences between the two strains of inbred mice were assessed by Student's *t*-test or by Mann-Whitney U-test. Analyses were performed with SuperANOVA software (Abacus Concepts, Berkeley, CA). Employing linear regression analyses, parameters significantly associated with the percent of the Ch absorption were further assessed by a stepwise multiple regression analysis to identify the independence of the association. Statistical significance was defined as a two-tailed probability of less than 0.05.

RESULTS

Absorption and lymphatic transport of radiolabeled Ch and palmitic acid

When conscious mice were kept in restraining cages with free access to 0.9% NaCl, basal lymph flow rates were similar in AKR mice (259 ± 43 μl/h) and C57L mice (262 ± 51 μl/h). Furthermore, with a continuous intraduodenal infusion of 0.9% NaCl or MCT with 0.5% TC (300 μl/h), lymph flow (**Fig. 1**, top panel) was increased to 275–310 μl/h in both AKR and C57L mice. We observed that in the absence of intraduodenal infusions of 0.9% NaCl or the lipid mixture containing 0.5% TC and MCT, most animals had to be eliminated from the 12 h study because of lymph clots, which decreased or stopped lymph flow. We found that the simple lipid mixture with TC in MCT performed much better than 0.9% NaCl, since it produced a steady and continuous lymph flow for longer periods of time. Figure 1 (bottom panel) shows the cumulative lymphatic transport of Ch and palmitic acid over 12 h in mice with intact biliary lipid secretion. Most radiolabeled Ch (70% in C57L mice and 62% in AKR mice) and palmitic acid (75–80% in both C57L and AKR mice) was recovered in intestinal lymph within the first 6 h, with the remainder being collected between 7 h and 12 h. Furthermore, cumulative radioactivities reached steady states by 12 h, with 95 ± 6% of the instilled [^{3}H]palmitic acid being recovered in lymph in the case of both strains of mice (P = NS). In contrast, 33 ± 6% of the instilled [^{14}C]Ch dose was recovered in lymph of C57L mice, and 21 ± 5% in lymph of AKR mice ($P < 0.05$). This implies that lymphatic Ch transport occurs mainly in the early stages of intestinal Ch absorption, there is no relationship between lymph flow and lymphatic Ch and palmitic acid transport,

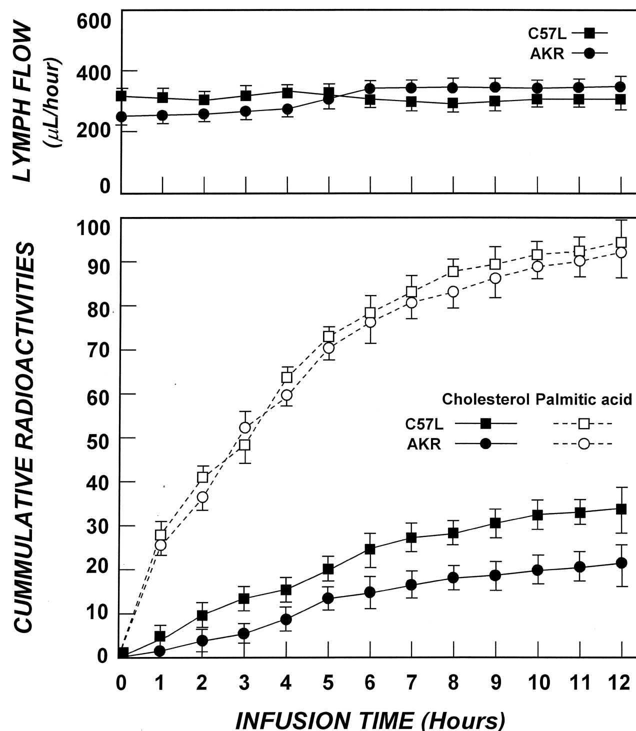

Fig. 1. Representative data (mean ± SD) for lymphatic transport of cholesterol (Ch) in AKR and C57L mice (n = 5 each), each with a continuous intraduodenal infusion at 300 μl/h of medium-chain triglyceride (MCT) mixed with 0.5% taurocholate. Top panel shows that lymph flow rates are constant and similar in both mouse strains over the 12 h period. The bottom panel displays cumulative radioactivities of [^{14}C]Ch and [^{3}H]palmitic acid recovered in mouse lymph as functions of time (h) after intraduodenal instillation of radiolabeled isotopes in animals with intact biliary lipid secretions. By 12 h, cumulative radioactivities of Ch and palmitic acid reach a steady state as shown by the closed and open symbols. Approximately 33 ± 3% of the instilled [^{14}C]Ch dose is recovered in lymph of C57L mice and 21 ± 3% in AKR mice ($P < 0.05$). The absorption and lymphatic transport of palmitic acid (open symbols) is similar between AKR and C57L mice, and at 12 h, recovery is 95 ± 3% of the dose.

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and C57L mice absorb fractionally more Ch than AKR mice, at least under these conditions.

Ch mass balance

Table 4 summarizes the data for daily Ch intake, daily biliary Ch output, daily fecal total neutral steroid excretion, daily absorbed Ch, and percent Ch absorption in inbred AKR and C57L mice in the metabolic steady state. Since the amount of food consumed was carefully quantified, the daily Ch intake could be analyzed by measuring Ch content of the diet by HPLC. We found that the lower-absorbing AKR mice and the higher-absorbing C57L mice ate identical amounts of Ch (0.84 ± 0.04 mg/day). However, daily biliary Ch outputs were significantly ($P < 0.0001$) higher in C57L mice (2.10 ± 0.07 mg/day) compared with AKR mice (1.13 ± 0.09 mg/day). Due to higher biliary Ch secretion, daily fecal total neutral steroid excretion was increased significantly ($P < 0.0001$) in C57L mice (2.61 ± 0.10 mg/day) compared with AKR mice (1.74 ± 0.11 mg/day). Nonetheless, an input-output analysis showed that the absorbed mass of Ch daily was significantly ($P < 0.001$) higher in C57L mice (0.32 ± 0.02 mg/day) compared with AKR mice (0.23 ± 0.03 mg/day). Using Eq. 1, the calculated percent Ch absorption in AKR mice was 27 ± 4%, significantly ($P < 0.01$) lower than that in C57L mice (39 ± 3%). Our results show that in a metabolic steady state, C57L mice absorb more Ch than AKR mice by the mass balance method.

Plasma radioactivities after ig administration of [^{14}C]Ch and iv injection of [^{3}H]Ch

Figure 2 demonstrates the changes over 120 h in plasma radioactivities measured at frequent intervals after ig administration of [^{14}C]Ch and iv injection of [^{3}H]Ch. Clearly, curves for iv [^{3}H]Ch were similar for AKR and C57L mice. They increased sharply over 6–12 h and reached their highest values in 12–24 h. Thereafter, plasma radioactivities decreased gradually and reached the lowest values at 120 h. Plasma radioactivities of ig [^{14}C]Ch began to rise over 3–12 h, and reached their highest values at 24 h, consistent with [^{14}C]Ch being absorbed from the small intestine to enter the circulation. It is apparent that [^{14}C]Ch counts decreased gradually over 48–120 h, approaching a point ~50% less than iv [^{3}H]Ch. The plasma distribution patterns between the ig-administered [^{14}C]Ch and the iv-injected [^{3}H]Ch were similar, despite the fact of both radioisotopes being given by different routes. Since at all time points, [^{14}C]Ch curves by ig dosing were higher in C57L mice than in AKR mice, this suggests that even by this indirect method, C57L mice absorb more Ch from the intestine than AKR mice.

Based on the ratios of two radioisotopes in plasma, we found that the apparent Ch absorption levels varied from 51 ± 7% at 24 h, 37 ± 5% at 72 h, and 49 ± 6% at 120 h in C57L mice, compared with 38 ± 6% at 24 h, 24 ± 4% at 72 h, and 36 ± 5% at 120 h in AKR mice. The Ch absorption values measured at 72 h by the plasma dual-isotope ratio method were closest to the levels of Ch absorption determined by the lymphatic transport and sterol balance methods, thereby validating a 3 day sampling time.

Food intake, fecal output, and excretion of radioisotopes after a single oral administration of radiolabeled Ch and sitostanol

Figure 3 shows the variations in daily food intake (top panel), fecal output (middle panel), and radioactivities recovered in feces (bottom panel) in AKR and C57L mice after an oral dose of [^{14}C]Ch and [^{3}H]sitostanol in MCT during the 10 day experimental period. It is apparent that both strains ate almost the same amounts of food, which varied between 3.5 and 4.4 g/day (Fig. 3, top panel), and displayed similar fecal outputs, ~1.3 to 1.6 g/day (Fig. 3, middle panel). Furthermore, no animal suffered from di-

TABLE 4. Ch balance data in AKR and C57L mice during metabolic steady-state conditions

Mouse	Ch Intake	Biliary Ch	Steroid Excretion	Absorbed Ch[a]	Ch Absorption[b]
	mg/day	*mg/day*	*mg/day*	*mg/day*	%
AKR mice					
1	0.82	1.08	1.64	0.26	32
2	0.86	1.10	1.74	0.22	26
3	0.84	1.25	1.84	0.25	30
4	0.86	1.19	1.85	0.20	23
5	0.81	1.01	1.61	0.21	26
Mean ± SD	0.84 ± 0.02	1.13 ± 0.09	1.74 ± 0.11	0.23 ± 0.03	27 ± 4
C57L mice					
1	0.81	2.01	2.47	0.35	43
2	0.83	2.15	2.66	0.32	39
3	0.89	2.18	2.75	0.32	36
4	0.80	2.11	2.58	0.33	41
5	0.85	2.06	2.61	0.30	35
Mean ± SD	0.84 ± 0.02	2.10 ± 0.07[c]	2.61 ± 0.10[c]	0.32 ± 0.02[d]	39 ± 3[e]

[a] Absorbed Ch was determined by subtracting the daily fecal neutral steroid output from the daily Ch intake and the daily biliary Ch output as measured by the HPLC method.

[b] The percent Ch absorption was calculated using Eq. 1 (see Materials and Methods).

[c] $P < 0.0001$ compared with AKR mice.

[d] $P < 0.001$ compared with AKR mice.

[e] $P < 0.01$ compared with AKR mice.

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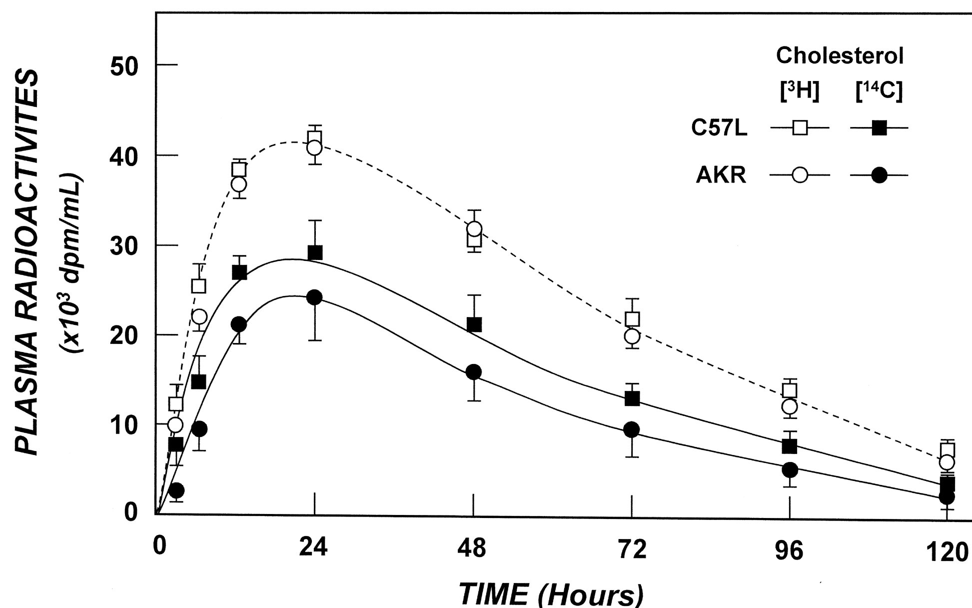

Fig. 2. Plasma radioactivities as functions of time (h) after iv injection of [^{3}H]Ch and ig administration of [^{14}C]Ch to AKR and C57L mice. Both plasma ^{3}H and [^{14}C]Ch radioactivities increase markedly during 3–12 h, and reach their highest values at 24 h. Thereafter, radioactivities decrease gradually and in parallel to reach their lowest points at 120 h. Furthermore, the iv injection curves of [^{3}H]Ch (open symbols) are identical between AKR and C57L mice. In contrast, the curves of [^{14}C]Ch (closed symbols) are significantly higher in C57L mice than in AKR mice at all time points. These data are consistent with the concept that C57L mice absorb more Ch from the intestine than AKR mice.

arrhea after ig administration of 150 μl of oil or skim milk vehicles. Shown in the bottom panel (Fig. 3) are the daily fecal excretions of [^{3}H]sitostanol and [^{14}C]Ch and their metabolites in AKR and C57L mice, respectively. The [^{3}H]sitostanol curves, which were similar between AKR and C57L mice, peaked at day 1, and the radiocounts were eliminated after day 4. Therefore, we chose the 4 day collection period for studying Ch absorption by the fecal isotope ratio method. In contrast, [^{14}C]Ch output curves peaked at day 1 and then decreased gradually over 10 days, which, at all time points, were markedly higher in AKR than in C57L mice. We assume that "Ch" radiocounts in feces (Fig. 3) after 4 days represents recirculation through the intestinal lymph to plasma and bile as Ch and its hepatic catabolic products the bile salts. These radiocounts were immaterial for evaluating Ch absorption, since no sitostanol counts remained in feces at these (>4 days) time points. Furthermore, compared with the 4 day pooled sample, apparent percent Ch absorption was increased by 26–30% in the 1 day sample and 8–16% in the 2 and 3 day pooled samples in both AKR and C57L mice. In contrast, in both strains of mice, apparent Ch absorption was decreased by 6–20% in the 6 and 10 day pooled samples. To study whether the entero(systemic)hepatic circulation of radiolabeled Ch influences measurement of Ch absorption, we measured radioactivities recovered in hepatic biles during the first 2 h of collection in different groups of AKR and C57L mice (n = 2–3 per strain). We carried out these experiments from day 0 to day 10 after ig gavage of 150 μl of MCT containing 1 μCi of [^{14}C]Ch. At day 2 and 3, only trace amounts of radiolabeled sterols were detected (inset in Fig. 3). Subsequently, the radioactivities recovered in hepatic biles peaked between days 4 and 5. After day 6, radiocounts in hepatic bile decreased markedly, and at day 10, the radioisotopes in hepatic bile were only minimally detectable. It is highly likely that radioisotopes in hepatic bile do not influence appreciably the measurement of Ch absorption efficiency by the fecal dual-isotope ratio method based on 4 day pooled feces.

Cumulative radioactivities in feces after a single oral dose of [^{14}C]Ch and [^{3}H]sitostanol

Figure 4 displays the cumulative radioactivities of [^{14}C]Ch and [^{3}H]sitostanol and their metabolites in feces over 4 days after a single oral dose. Fecal excretory patterns of [^{3}H]sitostanol were similar between AKR and C57L mice, with 82–88% recovery by day 1. The remainder (9–15%) was excreted over 2 to 4 days. Overall, the fecal recovery of sitostanol was 97 ± 4% in both AKR and C57L mice during the 4 day period. With respect to Ch, cumulative radioactivities at day 1 were 40 ± 4% in C57L mice and 50 ± 6% in AKR mice (P = NS); however, by day 4, the recovery was significantly ($P < 0.05$) higher in AKR mice (73 ± 5%) than in C57L mice (59 ± 5%). Clearly, the differences in ^{14}C-^{3}H ratios between the fed and fecal sterols was a measure of the differential absorption of the two sterols in each strain. We found that the levels of Ch absorption, calculated from the ^{14}C-^{3}H ratios of the dosing mixture and the 4 day pooled fecal samples, were 42 ± 7% in C57L mice and 29 ± 5% in AKR mice. Also, the levels of

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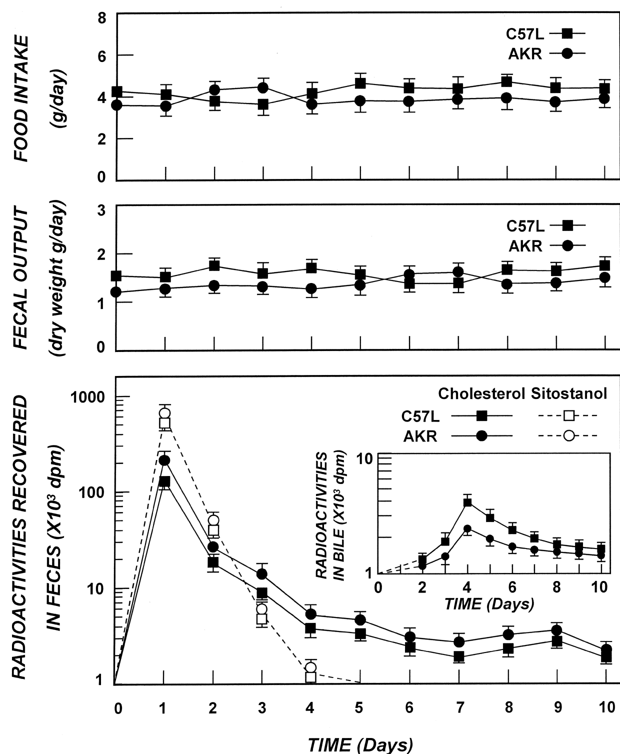

Fig. 3. Time dependence of food intake (top panel), fecal output (middle panel), and radioactivities of Ch and sitostanol recovered in feces (bottom panel). We investigated changes in these measurements in AKR and C57L mice over a 10 day experimental period. Each point represents the mean ± SD of 10 mice per strain. There are no significant differences in food intake or fecal outputs between AKR and C57L mice as functions of time (days). Both radioisotopes peak in the first day and decrease markedly during the second and third days. Only traces of [^{3}H]sitostanol are detectable by the fourth day, and tritium counts disappear completely in feces on the fifth day. [^{14}C]Ch and its metabolites are present in feces collected over the 4 to 10 day period. The [^{3}H]sitostanol curves are similar between AKR and C57L mice, whereas the [^{14}C]Ch curves are significantly higher in AKR mice compared with C57L mice at all time points. On the basis of these data, C57L mice clearly absorb more Ch from the small intestine than AKR mice. To study whether the entero(systemic)hepatic circulation of radiolabeled Ch influences measurement of Ch absorption, we investigated radioactivities recovered in hepatic biles in different groups of AKR and C57L mice (n = 2–3 per strain) from day 0 to day 10 after ig gavage of 150 μl MCT containing 1 μCi of [^{14}C]Ch. We found (inset, bottom panel) that radioactivities in hepatic biles peak between days 4 and 5. Since there are only trace amounts of isotopes, it is highly unlikely that Ch absorption efficiency is influenced based on measurements utilizing 4 day pooled feces.

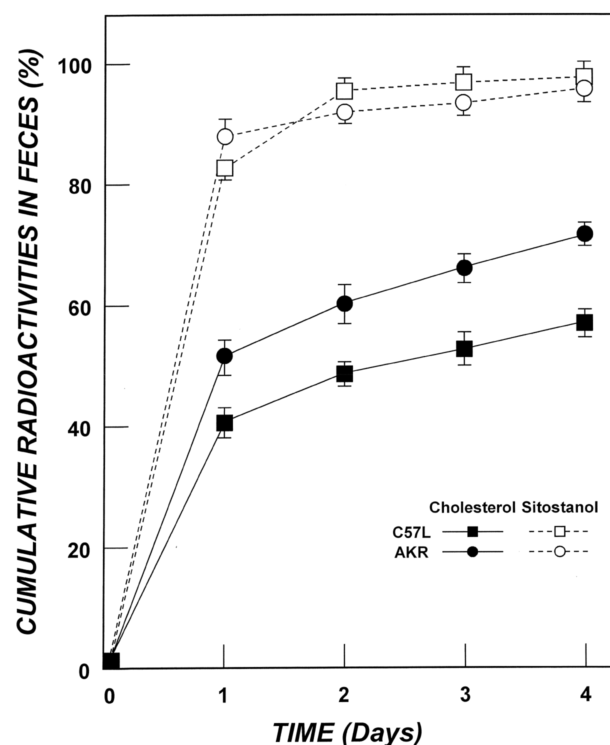

Fig. 4. Plots of cumulative Ch radioactivities and those of sitostanol recovered in mouse feces as functions of time (days). Each point represents the mean ± SD of 10 mice per strain. Excretory patterns of [^{3}H]sitostanol in feces (open symbols) are identical between AKR and C57L mice, with 97 ± 4% of sitostanol recovered from feces in both strains of mice during the 4 day period. In contrast, the final 4 day fecal recovery of radiolabeled Ch is 59 ± 5% in C57L mice and 73 ± 5% in AKR mice, suggesting that C57L mice absorb more Ch from the small intestine than do AKR mice.

Ch absorption determined by cumulative lymph Ch collection (direct measurement) at 12 h (Fig. 1) are similar to the values determined by plasma (37 ± 5% in C57L mice and 24 ± 4% in AKR mice) and fecal (42 ± 7% in C57L mice and 29 ± 5% in AKR mice) dual-isotope ratio methods (indirect measurements). These results show that there is excellent agreement among the three methods for measuring intestinal Ch absorption employing the time points indicated.

Influence of the delivery vehicle on Ch absorption efficiency

To study the influence of different delivery vehicles on Ch absorption efficiency, we examined chow-fed C57L mice that were given ig mixtures of [^{14}C]Ch (1/60 μmol) and [^{3}H]sitostanol (1/25 μmol) in 150 μl of various oils or skim milk (see Materials and Methods). **Figure 5** shows the variations in percent Ch absorption, measured by the fecal dual-isotope ratio method, in C57L mice fed these different vehicles. The lowest Ch absorption was found in mice fed corn (28 ± 6%) or soybean oils (29 ± 6%). In contrast, the animals fed sunflower oil (48 ± 7%), olive oil (53 ± 8%), safflower oil (57 ± 6%), or skim milk (65 ± 6%) displayed the highest levels of Ch absorption, with the groups fed peanut oil (40 ± 5%), rapeseed oil (41 ± 6%), or MCT oil (42 ± 7%) showing intermediate values.

We then compared the effects of MCT oil with corn oil and safflower oil on intestinal Ch absorption determined by the plasma dual-isotope ratio method. After three groups of mice received an iv injection of 2 μCi of [^{3}H]Ch in 100 μl intralipid, they were given an ig bolus of 1 μCi of [^{14}C]Ch in 150 μl of each vehicle. Again, the corn oil group (25 ± 4%) displayed the lowest percent Ch absorption, the safflower oil group (50 ± 6%) produced the highest Ch absorption, and the MCT oil group (37 ± 5%)

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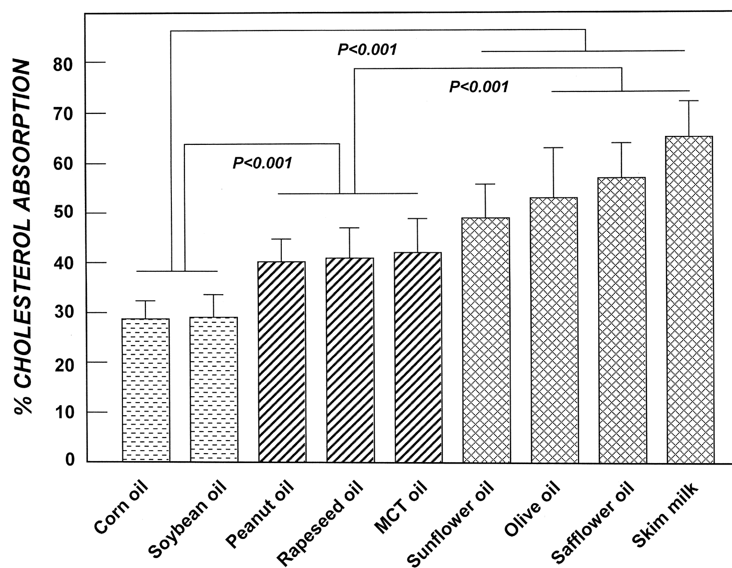

Fig. 5. Comparison of the effects of delivery vehicles for ig administration on percent Ch absorption in chow-fed C57L mice (n = 10 per group) determined by the fecal dual-isotope ratio method. Using 4 day pooled fecal samples, the percent of Ch absorption is lowest in mice fed corn (28 ± 6%) or soybean oils (29 ± 6%); intermediate in the groups dosed with peanut oil (40 ± 5%), rapeseed oil (41 ± 6%), or MCT oil (42 ± 7%); and highest in animals fed sunflower oil (48 ± 7%), olive oil (53 ± 8%), safflower oil (57 ± 6%), or skim milk (65 ± 6%). Significant statistical differences between low, mild, and high absorbers are shown.

gave an intermediate value. These findings demonstrate that the vehicles used for ig administration of isotopes influence Ch absorption efficiency, but they show similar effect on Ch absorption determined either by the plasma or fecal dual-isotope ratio methods.

Effects of feeding Ch and bile acids on intestinal Ch absorption

Figure 6 shows percent Ch absorption measured by the plasma dual-isotope ratio method in C57L mice fed Ch and bile acids. Compared with chow (37 ± 6%), there were no significant differences in Ch absorption in mice fed 0.5% Ch (33 ± 5%) or 1% Ch (29 ± 6%). However, feeding 2% Ch (22 ± 4%) and 0.5% UDCA (19 ± 3%) decreased efficiency of Ch absorption significantly ($P < 0.05$) compared with chow or the lower levels of Ch intake. In contrast, Ch absorption was increased significantly ($P < 0.001$) to 63 ± 7% in mice fed 0.5% CA alone, and to 55 ± 5% in mice fed the lithogenic diet that contained 0.5% CA. **Table 5** lists the compositions of bile salt species in individual hepatic biles of mice ingesting the different diets. All bile salts are taurine conjugated, and

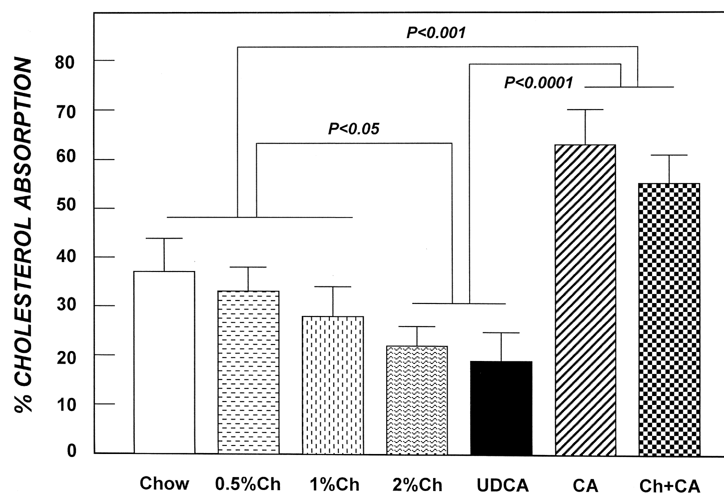

Fig. 6. Percent Ch absorption in C57L mice (n = 10 per group) determined by the plasma dual-isotope ratio method as functions of 0.02%, 0.5%, 1%, or 2% Ch, 0.5% ursodeoxycholic acid (UDCA), 0.5% cholic acid (CA), or 1% Ch plus 0.5% CA (lithogenic diet) feeding. Compared with mice fed chow (37 ± 6%), those fed 0.5% Ch (33 ± 5%) or 1% Ch (29 ± 6%) displayed similar percent Ch absorption efficiencies, but the values decrease significantly ($P < 0.05$) in mice fed 2% Ch (22 ± 4%). In general, the apparent percent Ch absorption decreases upon feeding a high-Ch diet because of dilution of the radiotracer as well as upregulation of intestinal ATP binding cassette efflux transporters. Feeding UDCA significantly ($P < 0.05$) decreases Ch absorption (19 ± 3%) compared with other native and added bile acids. Although mice fed CA (63 ± 7%) or the lithogenic diet (55 ± 5%) display significant ($P < 0.001$) increases in intestinal Ch absorption compared with chow, the percent Ch absorption in mice fed 0.5% CA alone was slightly higher, but was not significantly (P = NS) different from mice fed 1% Ch plus 0.5% CA.

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TABLE 5. Percent bile salt species in hepatic biles

Diet	TC	T-β-MC	TCDC	T-ω-MC	TUDC	TDC	HI[a]
Chow	50.1 ± 6.5	43.2 ± 5.6	0.9 ± 0.3	1.2 ± 0.5	2.1 ± 1.3	2.5 ± 1.1	−0.34 ± 0.03
0.5% Ch	49.6 ± 4.8	43.7 ± 3.5	1.2 ± 1.6	1.1 ± 0.9	1.9 ± 1.7	2.4 ± 1.4	−0.34 ± 0.04
1% Ch	45.2 ± 5.2	43.9 ± 4.1	1.9 ± 0.7	1.4 ± 1.0	2.6 ± 0.9	4.9 ± 0.8	−0.33 ± 0.02
2% Ch	44.6 ± 6.5	44.9 ± 4.0	2.0 ± 0.6	1.3 ± 0.8	2.1 ± 1.1	5.1 ± 1.9	−0.33 ± 0.03
0.5% UDCA	0.8 ± 0.4[b]	9.6 ± 2.6[b]	2.1 ± 0.7	1.7 ± 0.4	85.5 ± 4.5[b]	0.3 ± 0.1	−0.48 ± 0.04
0.5% CA	84.2 ± 5.1[b]	3.5 ± 1.9[b]	8.3 ± 4.4	0.9 ± 0.6	1.2 ± 1.0	1.8 ± 0.7	+0.01 ± 0.01[d]
1% Ch + 0.5% CA	72.5 ± 7.2[b]	6.3 ± 2.1[b]	11.2 ± 3.6[c]	0.8 ± 0.3	2.6 ± 1.6	6.5 ± 2.1	+0.02 ± 0.01[d]

CA, cholic acid; HI, hydrophobicity index; T-β-MC, tauro-β-muricholate; TC, taurocholate; TCDC, taurochenodeoxycholate; T-ω-MC, tauro-ω-muricholate; TDC, taurodeoxycholate; TUDC, tauroursodeoxycholate; UDCA, ursodeoxycholic acid. Vaues were determined in five individual hepatic biles (during first hour of biliary secretion) per mouse group.

[a] The HI values of five hepatic biles were calculated by Heuman's method (88).

[b] $P < 0.001$ compared with chow.

[c] $P < 0.01$ compared with chow.

[d] $P < 0.05$ compared with chow, Ch, or UDCA feeding.

the predominant species in mice fed chow and 0.5 to 2% Ch were TC (range 44.6–50.1%) and tauro-β-muricholate (T-β-MC) (range 43.2–44.9%), T-ω-MC (1.1–1.4%), tauroursodeoxycholate (TUDC) (1.9–2.6%), taurochenodeoxycholate (0.9–2.0%), and taurodeoxycholate (2.4–5.1%) being present in appreciably smaller concentrations. Feeding UDCA significantly increased TUDC (86%), whereas mice fed 0.5% CA with or without Ch displayed a strong increase in TC (72.5–84.2%) and a significant decrease in concentrations of the hydrophilic T-β-MC (3.5–6.3%).

Figure 7 illustrates that the percent of Ch absorption correlates significantly and positively with hydrophobicity indices (left panel) of the bile salt pool ($r = 0.94$, $P < 0.001$) and biliary Ch outputs (right panel) ($r = 0.81$, $P < 0.01$). Feeding the lithogenic diet and CA alone significantly ($P < 0.01$) increased not only the hydrophobicity indices of the bile salt pool, as well as biliary Ch outputs, but also Ch absorption levels compared with feeding chow. In contrast, the opposite effect occurred in mice fed UDCA. Upon feeding high Ch (0.5% to 2%) diets, the absorption of the radiotracer was decreased compared with chow feeding (Figs. 6; 7, left panel). The influence of Ch-containing diets is likely to be related to dilution of the radiotracer by the large intestinal pools of unlabeled Ch, as well as upregulation of the enterocyte sterol efflux transporters ATP binding cassette transporter g5 (Abcg5), Abcg8, and Abca1.

DISCUSSION

An accurate measurement of intestinal Ch absorption is one of the basic requirements for the quantitation of Ch homeostasis, and such experiments have been performed extensively in animals (4–7, 9, 13, 14, 16–38) and humans (8, 10–12, 15, 39–48) (Table 1). However, the published values for Ch absorption efficiency in mice span an enormous range varying from 20% to 90% (Table 2). These have been determined mostly by the fecal dual-isotope ratio method (6, 49–75), with fewer (5, 6, 75–79) estimated by the plasma dual-isotope ratio method (Table 3). The purpose of this study was to perform a systematic analysis of the three methods for the indirect measurement of intestinal Ch absorption and compare them with the direct lymph fistula method in mice. Accordingly, in the present study, the most important findings were: *i*) these methods are valid for measuring Ch absorption in mice, with excellent agreement between the three indirect methods as measured in plasma samples taken on the third day, four day pooled feces, and in the metabolic steady state, as well as the direct approach by 12 h intestinal lymph collections; *ii*) the solubilizing vehicles used for ig administration of radioisotopes influence the levels of Ch absorption markedly; and *iii*) percent Ch absorption correlates positively with the hydrophobicity indices of the bile salt pool and with the levels of biliary Ch outputs due to increased Ch solubilization in micelles, but negatively with exogenous Ch intake because of radiotracer dilution. We believe that when methodological variations are carefully controlled for, genetic differences may be quantified for analysis of interstrain variations in Ch absorption efficiency.

Reevaluation of methods for measurement of intestinal Ch absorption

The most direct method for determining Ch absorption is measurement of Ch that is transported from the intestinal lumen into the mesenteric or thoracic lymph duct. This requires cannulation of the lymphatic duct and insertion of a duodenal cannula for infusion of radiolabeled Ch. In our in situ experimental system, mice were anesthetized during the experiments, which allowed hourly collection of fresh lymph samples without the effects of stress on the animals such as could be induced by restraining cages (89, 90). A critical factor in the validity of the Ch absorption measurement by lymph fistula is maintenance of constant lymph flow during the study. Furthermore, estimation of radiolabeled fatty acid absorption is an additional test (91) to evaluate the success of lymph duct cannulation as well as lymph Ch transport, because *tracer* fatty acids, even saturated ones such as palmitic acid, are absorbed completely by the small intestine (92). At 12 h, cumulative Ch radioactivities in lymph reached a steady state, with 95% of the infused fatty acid being recovered in lymph of both mouse strains (Fig. 1). Therefore, cumulative radioactivities from 12 h lymph collections repre-

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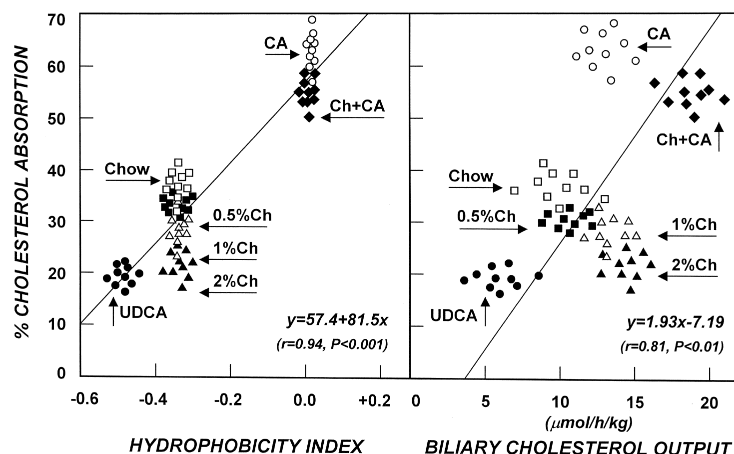

Fig. 7. Percent Ch absorption as functions of the hydrophobicity of biliary bile salt pool (left panel) and the unperturbed biliary Ch outputs (right panel) in C57L mice (n = 10 per group) fed chow (Ch < 0.02%), 0.5%, 1%, or 2% Ch, 0.5% UDCA, 0.5% CA, or the lithogenic diet containing 1% Ch and 0.5% CA for 1 week. Each point represents percent Ch absorption, hydrophobicity indices of hepatic biles, and biliary Ch outputs in the same mouse. Ch absorption efficiency correlates significantly ($P < 0.001$) and positively ($r = 0.94$) with hydrophobicity indices of the bile salt pool (left panel), and significantly ($P < 0.01$, $r = 0.81$) with biliary Ch outputs (right panel). This suggests that higher bile salt hydrophobicity indices enhance, and higher biliary Ch outputs are associated with, intestinal Ch absorption. However, the latter may be the result rather than the cause of augmented absorption. Moreover, increasing dietary Ch apparently reduces the percent of Ch absorption (left panel) due to dilution of the radiolabeled Ch by the large intestinal pools of unlabeled Ch, and up-regulation of the enterocyte Ch efflux transporters Abcg5, Abcg8, and Abca1. Closed square, animals were fed chow; open square, 0.5% Ch; open triangle, 1% Ch; closed triangle, 2% Ch; closed circle, 0.5% UDCA; open circle, 0.5% CA; diamond, the lithogenic diet containing 1% Ch and 0.5% CA.

sent precise Ch absorption values in mice. Also, it has been observed that the absorption and lymphatic transport of Ch is complete by 10–12 h in rats (14, 16, 17), rabbits (23, 24), dogs (28, 29), primates (31, 32), and humans (15, 39, 40).

Sterol balance methods depend upon the precise analysis and administration of dietary constituents, as well as total collection of feces and accurate measurement of endogenous biliary Ch outputs and fecal neutral steroids. In this study, the metabolic steady state is arbitrarily defined by constant food intake, fecal excretion of steroids, and body weight during the period of study. The two most important advantages of this method are that radioactive materials are not necessary, and it is more physiological with more facile surgery than the lymphatic transport method, although the sterol balance approach requires measurement of biliary Ch outputs in an anesthetized mouse.

The fecal dual-isotope ratio method introduced by Borgström (7) involves the ig administration of radiolabeled Ch and a nonabsorbed β-sitosterol (7, 8) or sitostanol (9). Since this method calculates absorbed Ch by the analysis of nonabsorbed Ch in feces, any Ch "losses" during transit through the gastrointestinal tract are measured as absorbed Ch. We observed that sitostanol is minimally absorbed in mice, with >97% of radiolabeled sitostanol being recovered in feces. Therefore, when compared with the Ch-sitostanol ratio administered orally, the ratio in the neutral steroid extract of feces should provide an accurate measurement of Ch absorption. We observed that a single fecal sample taken early, on the first day for example, leads to an overestimation of Ch absorption by 26–30%. Other investigators also found that the ratio of Ch-sitostanol in feces is high initially and decreases throughout the test period in rats (7), rabbits (26), baboons (35), and humans (47). Therefore, the validity of this method depends upon the determination of the isotope ratio in an aliquot of pooled feces collected from day 0 until the time when sitostanol excretion becomes negligible. If the fecal collection period is extended too long, the percent of Ch absorption is underestimated due to recirculation of radioactive Ch as itself and bile salts. We proved this point and found in the present work that an appropriate time period for pooling fecal collections is 4 days in mice. It has been reported that Ch absorption can be measured precisely using 4 day pooled feces in rats (7) and hamsters (9), yet 5 day pooled feces is required in guinea pigs (22) and rabbits (26), and 7–8 day pooled feces is necessary for baboons (35), monkeys (37) and humans (47). Because daily frequency of large intestinal evacuation is quite different between rodents (mice, rats, hamsters, guinea pigs, and rabbits) and higher mammals (baboons, monkeys, and humans), it is apparent that higher mammals have slower large intestinal transit times compared to rodents. Therefore, employing this method, it is necessary to collect 7 day pooled feces for measuring the efficiency of intestinal Ch absorption in primates, including humans.

The plasma dual-isotope ratio method is the simplest technique developed first by Zilversmit (4) for use in rats, and its utility has been extended to other animal species and humans (Table 1). The validity of this method re-

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quires that the radioactivities time curves of the two isotopes in plasma are parallel (18, 93). We observed that this occurs at 3 days in mice (Fig. 3). However, it has been found that parallelism begins at 2 days in rats (13), at 3 days in hamsters (21), and between 3 and 4 days in monkeys (36) and humans (44). It should be emphasized that this method becomes invalid if the absorbed portion of an ig-administered dose is not distributed in the same manner as an iv-injected dose. For example, rabbits fail to release an iv Ch dose from tissues into plasma even over an extended period, so percent Ch absorption is overestimated by this method (25).

Taken together, our results show that no significant differences are evident in the efficiency of intestinal Ch absorption measured between the three indirect methods and the direct 12 h lymph collection method. The excellent agreement observed between all four methods also indicates that gallstone-susceptible C57L mice absorb significantly more Ch from the small intestine than gallstone-resistant AKR mice (6).

Comparison of murine Ch absorption efficiency with that of other animal species

Table 1 summarizes Ch absorption rates in different animal species and humans from the literature. For the sake of uniformity, the data cited are limited to those using one of the methods studied in the present work. It is clear that Ch absorption efficiency in animals shows considerable species variability. Rabbits and dogs absorb particularly large amounts of dietary Ch with a corresponding tendency toward diet-induced hypercholesterolemia (28, 29, 94). While generally demonstrating a dose-response relationship to Ch feeding, rats, monkeys, and humans absorb lower percentages of dietary Ch, and some primates, including humans, show considerable resistance to Ch feeding (94). Actually, mice are also highly resistant to Ch feeding in the natural state, and they have a more hydrophilic bile salt pool with, typically, equimolar amounts of T-β-MC and TC (5, 6, 82, 83). The reason for modest Ch absorption from the small intestine is that micellar T-β-MC is a very poor Ch solubilizer with similar properties to the taurine conjugate of UDCA (95). However, humans have essentially no β-muricholates and little UDCA conjugates, and exhibit a more hydrophobic bile salt pool with high glycine and taurine conjugates of cholate (~35%), chenodeoxycholate (~40%), and deoxycholate (15%) (96). As we observed in this study, using the range (25% to 42%) of Ch absorption from the lowest (AKR strain) to the highest (C57L strain), mice absorb smaller quantities of Ch in the native state than do humans (35–55%) and rats (38–60%) (Table 1).

Technical considerations underlying variations in intestinal Ch absorption

The methods for measuring intestinal Ch absorption, when properly applied, should give convincing and comparable results. However, these techniques all suffer from numerous potential sources of error that can lead to divergent results. These sources of error relate, in part, to the collection times of plasma and feces, as well as improper techniques in the preparation of radioisotopes and their administration, the efficient extraction and saponification of radioisotopes from feces, and many technical aspects associated with mouse surgery. Different vehicles used by various laboratories (Tables 2, 3) are a leading factor increasing deviations in Ch absorption efficiency (Fig. 5), as we will now discuss.

We observed that radiolabeled Ch and sitostanol should be dissolved in appropriate oily vehicles to prevent their phase separation as crystals in the oily vehicle or intestinal lumen. However, radioisotopes mixed with chow, skim milk, or 0.9% NaCl produced apparent increases in the levels of Ch absorption, possibly because they are not good vehicles to solubilize even traces of Ch (solubility Ch 10^{-8} M in H_2O). If the lipid mixture is vomited, or injected into soft tissues, this will clearly void the Ch absorption study. Overdoses of anesthetic agents may retard intestinal transit due to an extended recovery period. All of these could lead to artificially increased levels of Ch absorption, which are clearly nonphysiologic.

Early workers realized that isolation, extraction, and quantification of fecal neutral steroids are difficult and time-consuming experimental procedures (11, 12, 87). Miettinen et al. (11, 12) developed a series of analytical methods for quantifying fecal neutral steroids, which are sufficiently sensitive to be employed in individual small laboratory animals. Borgström (87) proved further that fecal neutral steroids can be recovered completely from rat feces by the methods of Miettinen et al. (11, 12). Both of these groups (11, 12, 87) found that saponification of fecal neutral steroids is an important step. Most likely, radiolabeled Ch and sitostanol and their bacterial metabolites in the large intestine bind strongly to endogenous undigested mucins, dietary fibers, and bacterial membranes, as well as fatty-acid soaps, which could influence total recovery of radioisotopes from feces. Therefore, the Ahrens and Borgström laboratories (11, 12, 87) suggested saponification of fecal samples with (2N)NaOH-methanol (1:1, vol/vol) followed by incubation at 60°C for 1 h. Since the amount of esterified sterols in feces is small, this procedure most likely breaks the affinity of radioisotopes for absorbents, and possibly renders complete recovery of fecal neutral steroids, including radiolabeled Ch and sitostanol as well as their metabolites. The isolation and extraction of lipids by chloroform-methanol (2:1, v/v) (97) without "saponification" makes complete recovery of fecal neutral steroids highly problematic. Unfortunately, several laboratories have failed to notice the importance of saponification or have not validated their extraction methodology against this gold standard (11, 12, 87). It may explain, in part, why the percent Ch absorption is often overestimated by methods involving fecal collection.

To successfully perform cannulation of the mesenteric lymphatic duct, a key step is obviously to clearly identify and expose this structure. We also suggest that a continuous intraduodenal infusion of a lipid mixture be utilized to keep lymph flow constant during the fistulation. Previ-

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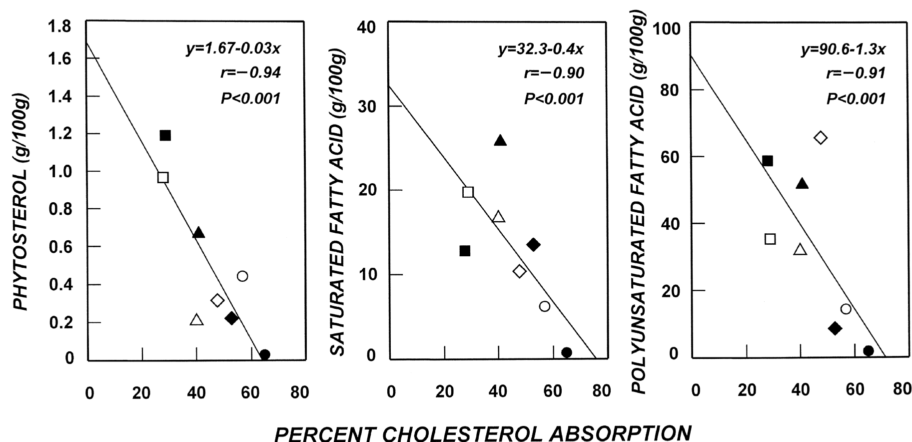

Fig. 8. Concentrations of total phytosterols, saturated, and polyunsaturated fatty acids of different commercial edible oils plotted against the percent Ch absorption observed in the present study. Data are based on the USDA database for standard reference compositions of nutrient oils (98) and the literature (99–102). Ch absorption efficiency varies significantly ($P < 0.001$) and inversely ($r = -0.90$ to -0.94) with the quantity of total phytosterols, as well as saturated and polyunsaturated fatty acids contained within the oils (98–102) used in this study. Not shown here are the concentrations of monounsaturated fatty acids plotted against percent Ch absorption, which demonstrates a scattered distribution. Open square, corn oil; closed square, soybean oil; open triangle, peanut oil; closed triangle, rapeseed oil; open diamond, sunflower oil; closed diamond, olive oil; open circle, safflower oil; and closed circle, skim milk (control).

ously, lymphatic transport of Ch was measured in conscious small animals, such as rats (14, 16, 17) and rabbits (23, 24), that were kept in restraining cages. Although there is no significant difference in lymph flow rates between anesthetized and conscious mice, we noted that conscious mice become too stressed to survive for the extended experimental periods required for steady-state lymph collections up to 12 h. Moreover, it is easier to maintain anesthetized mice in a physiologic state with iv infusion of 0.9% NaCl for hydration.

Different oily vehicles exhibit marked Ch absorption differences

We noted that the vehicles used for ig administration of radioisotopes influence the percent of Ch absorption markedly (Fig. 5), which is most likely related to different concentrations of total phytosterols, as well as triglyceride compositions of saturated and polyunsaturated fatty acids in these oils (98–102). Most oils triglyceride-rich in polyunsaturated fatty acids are naturally abundant in phytosterols (103), which decrease plasma Ch concentrations by inhibiting intestinal Ch absorption (103). **Figure 8** shows significant ($P < 0.001$) and negative ($r = -0.90$ to -0.94) relationships between percent Ch absorption and the concentrations of phytosterols (left panel), saturated (middle panel), and polyunsaturated (right panel) fatty acids contained in the oils studied (98–102). Because the constituent fatty acids of MCT oil have essentially a neutral effect on Ch metabolism (104, 105), this oil was selected as the vehicle for oral dosing procedures (9, 21). Turley et al. (21) examined the effect of MCT, olive, and safflower oils on Ch absorption in hamsters, and their limited results are essentially in agreement with ours, showing that the percent Ch absorption decreases in the rank order MCT oil < olive oil < safflower oil (Fig. 5).

Influence of bile salt hydrophobicity and dietary Ch on intestinal Ch absorption

Feeding bile acids changes the composition and hydrophilic-hydrophobic balance of the bile salt pool markedly (Table 5). This in turn regulates the level of Ch absorption (5, 106) due to their differential capacities for micellar Ch solubilization (95). We found that Ch absorption efficiency correlates significantly ($P < 0.001$) and positively ($r = 0.94$) with the hydrophobicity indices (88) of the bile salt pool (Fig. 7, left panel). Feeding CA renders the murine bile salt pool more hydrophobic because of an increase in TC and a decrease in T-β-MC concentrations (5, 82, 83), which in turn enhances intestinal Ch absorption (5). For this reason, cholelithogenesis cannot occur spontaneously in healthy inbred mice without the addition of 0.5% CA and 1% Ch to the mouse diet (82, 107). The diminished Ch absorption efficiency with oral UDCA is well documented in humans (108), and has also been reported in hamsters (109). In contrast, intestinal Ch absorption is also increased in hamsters and rats given CA (109, 110).

Akiyoshi and colleagues (62, 111) observed that there is a strong positive relationship between high intestinal Ch absorption and elevated biliary lipid secretion rates in rodents. We also found that the percent Ch absorption correlates positively with biliary Ch outputs (Fig. 7, right panel), suggesting a cause and effect relationship. Moreover, C57L mice essentially double their biliary Ch outputs compared with AKR mice (83). Our findings are not in agreement with the recent results of Sehayek et al. (55),

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who claimed that elevated biliary Ch secretion rates reduce intestinal Ch absorption in mice. Actually, they (55) studied Ch contents of mouse gallbladder biles, but did not examine biliary Ch outputs. It must be pointed out that compared with AKR mice, C57L mice (82, 83) also show significantly higher biliary bile salt and PL outputs, as well as greater sizes and hydrophobicity indices of bile salt pools, all of which may be responsible for enhancing Ch absorption.

In contrast, increased loads of dietary Ch, while increasing mass uptake by the enterocyte, decrease the efficiency of intestinal Ch absorption in animals, as assayed by fecal (55, 65) and plasma (21) dual-isotope ratio methods. Clearly, the percentage of Ch absorption is different from the values of total Ch mass absorbed. Whiting and Watts (65) calculated that total Ch mass absorbed in mice increases from 1.4 mg/day when ingesting chow (Ch $<$ 0.02%) to 21 mg/day in mice fed 1% Ch (total food intake = 5.7–6.8 g/day). Concomitantly, their results (65) showed that the percent Ch absorption of a radiolabeled tracer decreases from 61% in chow-fed mice to 31% in mice fed 1% Ch. We found that in each group (Fig. 6), the percent Ch absorption progressively decreased with increases in dietary Ch (0.02 to 2%), showing a significant negative relationship. The principal reasons for these differences are secondary to the effect of added Ch mass diluting the radioisotope in the upper small intestine. Of special note are the recent findings on the functions of intestinal sterol efflux transporters Abca1 in mice (112), and ABCG5 and ABCG8 in humans (113, 114). Upon feeding high Ch diets, ABC transporters efflux a substantial percentage of Ch that is absorbed by the enterocyte back into the intestinal lumen for excretion from the body. Of course, this could also occur for traces of radiolabeled Ch on a high-Ch diet. It should be emphasized that both plasma and fecal dual-isotope ratio techniques are not suitable methods for studying Ch absorption efficiency under conditions of a high Ch intake. Thus, the proper methods to utilize under these conditions should be Ch balance and lymph fistula approaches, since both can determine not only the percent of Ch absorption but also total mass of Ch absorbed from the small intestine.

In summary, our systematic studies on quantifying Ch absorption in mice establish excellent agreement between the indirect methods as analyzed in plasma samples taken on the third day, in 4 day pooled feces, in the metabolic steady state, and in the direct method as determined by 12 h cumulative mesenteric lymph collections. We prove herein that all four methods are valid for measurement of Ch absorption in mice. It should be pointed out that if the percent Ch absorption in healthy inbred mice fed chow is found to be more than 55% when measured by one of the four methods described, particularly using MCT as the delivery vehicle, it is likely that the results are overestimated. Moreover, other natural oily vehicles will influence percent Ch absorption depending on their phytosterol contents as well as the fatty-acid compositions and extent of unsaturation. Our methods should provide a basic framework for comparing Ch absorption from the intestine and homeostasis in inbred mouse studies between different laboratories, as well as for investigating candidate genes that regulate the absorptive processes. Most importantly, we suggest that the in situ lymphatic Ch transport method in anesthetized mice can be used for exploring lymphatic absorption of lipids and drugs, the assembly and secretion of chylomicrons, and the intestinal lipoprotein metabolism.

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