Bile acid production in human subjects: rate of oxidation of [24,25-^{3}H]cholesterol compared to fecal bile acid excretion

Nicholas O. Davidson,[1] H. Leon Bradlow, E. H. Ahrens, Jr., Robert S. Rosenfeld,* and Charles C. Schwartz**

The Rockefeller University, New York, NY 10021, Beth Israel Medical Center,* New York, NY 10003, and Medical College of Virginia,** Richmond, VA 23298

Abstract Bile acid production has been quantitated in seven subjects by methods that compare the results of two independent approaches, namely, quantitation of cholesterol side-chain oxidation and fecal bile acid excretion. Six hypertriglyceridemic (HT) subjects and one normolipidemic control were studied by both techniques. A further control subject was studied by the cholesterol side-chain oxidation method alone. Cholesterol side-chain oxidation was quantitated by measuring the appearance of ^3H_2O after intravenous administration of [24,25-^{3}H]cholesterol, using multicompartmental analysis of plasma cholesterol and [^{3}H]water specific activity. Body water kinetics were independently defined by use of oral D_2O. Two HT subjects were restudied while they were taking cholestyramine, 16 g/day. In all ten studies, multicompartmental analysis closely simulated the observed appearance of ^3H_2O. Values obtained for bile acid production suggest that cholesterol oxidation, or bile acid input, was significantly greater than fecal bile acid output in the HT subjects ($P < 0.05$). Cholesterol side-chain oxidation rates in the two normal subjects were lower than those encountered in HT subjects, being similar to published values for normal subjects both for bile acid synthesis as determined by isotope dilution kinetics and fecal bile acid excretion. Studies conducted with two, synthetically different, preparations of [24,25-^{3}H]cholesterol indicated that, in one of the two preparations, approximately 20% of the tritium label was at positions proximal to C_{24}. In the other preparation examined, all of the tritium was located at, or distal to, C_{24}. Further studies revealed that 0.055–0.24% of the dose was present as labile tritium by virtue of its appearance as ^3H_2O following in vitro incubation with human plasma. Provided these isotope effects are taken into account, multicompartmental analysis of plasma [24,25-^{3}H]cholesterol and body water appears to be a useful technique for quantitating cholesterol oxidation in human subjects. —**Davidson, N. O., H. L. Bradlow, E. H. Ahrens, Jr., R. S. Rosenfeld, and C. C. Schwartz.** Bile acid production in human subjects: rate of oxidation of [24,25-^{3}H]cholesterol compared to fecal bile acid excretion. *J. Lipid Res.* 1986. **27:** 183–195.

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Bile acid synthesis represents the most important route of degradative elimination of cholesterol from the body and its quantitation is a key parameter in cholesterol homeostasis. The biochemical transformation of cholesterol, a 27 carbon steroid, into cholic and chenodeoxycholic acids, both 24 carbon steroids, involves sequential nuclear and side chain modification of the steroid molecule by the liver. Upon cleavage of the C_{24-25} bond of cholesterol, during its ultimate conversion into primary bile acids, propionic acid is formed and rapidly converted to CO_2 and water (1). This phenomenon has been exploited by workers investigating bile acid synthesis rates in a variety of experimental animals (2–6) and in man (1, 7).

We have used multicompartmental analysis of plasma cholesterol specific activity, body water kinetics, and the magnitude of ^3H_2O appearance following intravenous administration of [24,25-^{3}H]cholesterol to measure bile acid production in human subjects. Having validated directly several key assumptions intrinsic to the use of side-chain labeled [^{3}H]cholesterol, we investigated six hypertriglyceridemic (HT) subjects and two normolipidemic controls. Two of the six HT subjects were restudied while they were taking cholestyramine, 16 g/day. The combined cholesterol and water models accurately predicted the pattern of ^3H_2O appearance in all subjects studied. The values for bile acid production by this method, however, were systematically larger than the corresponding values for fecal bile acid excretion in the

Abbreviations: HT, hypertriglyceridemia; D_2O, deuterium oxide; FRC, fractional rate constant; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

[1]Present address and author to whom communication should be directed: Gastrointestinal Unit, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.

hypertriglyceridemic patients; the two values agreed closely in the control subjects.

MATERIALS AND METHODS

Subjects

Seven subjects were admitted to the Metabolic Ward of Rockefeller University Hospital. Relevant clinical data are presented in **Table 1**. All were maintained on repetitive eucaloric diets, in some instances by liquid formula feeding (8, 9); in other cases with cyclic (2- or 3-day) solid food diets of measured protein, fat, and carbohydrate content. Details of caloric and dietary sterol intake are listed in Table 1. Following admission to the Metabolic Ward, and prior to the initiation of any study, patients were required to establish a metabolic steady state in which body weight was maintained constant ± 0.2 kg, biweekly plasma cholesterol and triglyceride determinations showed a coefficient of variation of less than 10% with no obvious trend, and recovery of chromic oxide from the stool (10) exceeded 80%. Two patients were studied twice; on the first occasion with no medication, on the second occasion after a new steady state had been achieved while taking cholestyramine, 8 g twice daily for 6 weeks prior to and during the study. A second normolipidemic control subject was studied as an outpatient. The subject maintained his body weight and caloric intake at a prescribed level for the duration of the study.

After review and approval of the study protocol by the Rockefeller University Institutional Review Board, informed consent was given by all patients.

TABLE 1. Relevant clinical data in eight study subjects

Patient	Age	Sex	Height	Weight	Relative Body Weight[a]	Diet	K-Calories[b]	Dietary Steroids[c]		Plasma Lipids[d]		Diagnosis[e]
								Chol.	Sito.	Chol.	Triglycerides	
	yr		*cm*	*kg*	%							
1 (a)[f]	68	F	159	81	139	Formula[g]	2200	34	588	368 ± 15 (17)	218 ± 21 (17)	CH, TX, IHD Type IIb
(b)	69	F	159	72.5	123	Formula	1900	29	508	265 ± 12 (18)	172 ± 12 (18)	As above plus cholestyramine 16 g/day
2 (a)	37	M	170	90	129	Solid[h]	2800	159	188	298 ± 17 (22)	646 ± 53 (22)	CH, IHD Type V, gout propranolol 200 mg/day allopurinol 300 mg/day
(b)	37	M	170	90	129	Solid	2800	159	188	310 ± 36 (17)	1087 ± 122 (17)	As above plus cholestyramine 16 g/day
3	52	M	168	72	106	Solid	2700	127	284	550 ± 24 (23)	199 ± 26 (21)	CH, TX, IHD Type IIb
4	51	M	169	67	97	Formula	2400	341	262	206 ± 43 (22)	482 ± 87 (22)	HG, IHD Type IV
5	67	F	165	75	115	Solid	2840	304	143	188 ± 9 (22)	311 ± 32 (22)	HG, IHD cholecystectomy Type IV
6	65	M	167	79.5	119	Solid	2400	257	121	329 ± 16 (21)	1111 ± 99 (21)	CH, IHD Type IV
7	60	M	174.5	67.5	91	Formula	2400	240	228	219 ± 6 (17)	179 ± 43 (17)	NL
8	66	M	174	76.6	104	Solid	2500			236 ± 14 (8)	146 (1)	NL, outpatient

[a] Relative body weight (% ideal body weight) = $\frac{\text{weight (kg)}}{\text{height (cm)} - 100} \times 100$.

[b] Daily energy intake required to maintain constant body weight: (1 kilocalorie = 4.184 kilojoules).

[c] Daily intake mg/day.

[d] Values given as mg/dl ± SD during metabolic steady state (see text); number of analyses in parentheses.

[e] Abbreviations: CH, combined hyperlipidemia; TX, tendon xanthomas; IHD, ischemic heart disease; NL, normal plasma lipid values. Figures in Roman numerals refer to phenotypic pattern by the Fredrickson, Levy, and Lees (50) classification.

[f] Letters (a) or (b) in parentheses refer to successive study periods in the same subject.

[g] Oral formula feedings with vitamin and mineral supplements (8, 9). Study subjects 1(a), 1(b) received 40% daily caloric intake as corn oil, 15% and 45% protein and carbohydrate, respectively. Study subject 4 received 35% daily caloric intake as cottonseed oil, 15% and 50% as protein and carbohydrate, respectively. Study subject 7 received 31% daily caloric intake as cottonseed oil, 15% and 54% protein and carbohydrate, respectively.

[h] Solid food diets were given in repetitive 2- or 3-cycles and were determined by analysis to furnish the same mixture of major nutrients, cholesterol, and plant sterols each day. Study subjects 2(a), 2(b) received 61% daily caloric intake as fat with a P/S ratio of 4.3/1, protein and carbohydrate 14% and 25%, respectively. Study subjects 3, 5, and 6 received 35% of their daily caloric intake as mixed food fats with a P/S ratio of 2/1; protein and carbohydrate comprised 20% and 45%, respectively.

Isotope preparation and administration

[24,25-^{3}H]Cholesterol was obtained from two different sources. The first, referred to as preparation #1 was a gift from Dr. Richard Hochberg (Roosevelt Hospital, New York, NY) and was prepared by reductive tritiation of desmosterol with tritium gas using triphenylphosphine-chlororhodium in dioxane as a catalyst (11). The second, referred to as preparation #2, was prepared by reduction of i-desmosterol-6-methyl ester with tritium gas using palladium black as a catalyst (7). [4-^{14}C]Cholesterol was obtained from New England Nuclear Corp., Boston, MA at a specific activity of 40 mCi/mmol. All preparations of radiolabeled cholesterol were subjected to preparative thin-layer chromatographic (TLC) separation using silica gel G in a solvent of benzene–ethyl acetate 4:1 (v/v). In addition, purification by reversed phase celite column chromatography was performed (11) and the [24,25-^{3}H]-cholesterol preparations were mixed with chemically authentic, freshly recrystallized cholesterol to a dilution of 5 mCi/mmol. Gas–liquid chromatography (GLC) analysis of each preparation confirmed a single (mass) peak with the appropriate retention time (relative to 5α-cholestane) of cholesterol. For the purposes of administration, [24,25-^{3}H]cholesterol was dissolved in 200 μl of absolute ethanol; the solution was then mixed with 2 ml of propylene glycol and administered through the side arm of a venoset infusion. The precise dose of tritiated cholesterol administered was derived by weighing the syringe before and after isotope administration and multiplying by the specific activity (dpm/g) of the infusate, which was separately determined. Patients were infused with approximately 60 μCi of [24,25-^{3}H]cholesterol (range, 30–90 μCi).

Biological reliability of the [24,25-^{3}H]cholesterol preparations

Biological reliability was determined by intravenous coadministration of each preparation of [24,25-^{3}H]cholesterol together with [4-^{14}C]cholesterol (12). These studies were conducted on six additional in-patients at the Rockefeller University Hospital, patients 9–14. As part of the investigation of tritium distribution (see below) within the labeled cholesterol molecule, each of these six patients was intubated with a nasoduodenal catheter which was positioned in the second portion of the duodenum under fluoroscopic control.

Sample collection and analysis

Fasting plasma samples and continuous 24-hr urine collections were obtained daily for 21 days following infusion of [24,25-^{3}H]cholesterol. Plasma (total) cholesterol specific activity was determined on measured aliquots following saponification and extraction into petroleum ether (bp 33–60°C) with mass measurements being made using Auto Analyzer II technology (Technicon Instruments Corp., Tarrytown, NY). Separate determinations of plasma free and esterified cholesterol were made in only one control subject (patient #8). In this subject, blood was drawn at 0, 24, 36, 48, 60, 72, 84, and 96 hr after isotope administration and measurements were made of both free and esterified cholesterol specific activity after separation by TLC following neutral sterol extraction, as previously described (7). Measurement of the specific activity of tritiated water in plasma and urine and atom % excess D_2O was carried out on lyophilized samples. Liquid scintillation spectrometry was conducted on aliquots of lyophilizate using Hydrofluor (National Diagnostics, Somerville, NJ) as the scintillant and a Packard tri-carb scintillation counter (model 3380-3390, Packard Instrument Co., Downer's Grove, IL) with quench corrections performed automatically by an absolute activity analyzer (AAA model 544, Packard Instrument Co.). Measurement of atom % excess D_2O was carried out by mass spectrometry and was graciously performed by Dr. Frank H. Field, Rockefeller University.

In studies, to be described below, where tritium distribution in the side chain of the two preparations of [24,25-^{3}H]cholesterol was examined, bile and plasma samples were simultaneously withdrawn at 48-hr intervals up to 20 days. Bile samples were added to ten volumes of absolute ethanol and refrigerated at 4°C until analyzed. Radioactivity and mass determinations were carried out on both the neutral and acidic steroid fraction of biliary lipid (13). Bile acid radioactivity measurements were carried out individually on cholic, chenodeoxycholic, and deoxycholic acids following TLC separation of the methyl ester derivatives (13). GLC measurement (1% High Eff-8 BP 5' column) of the eluted fraction from each of the three major biliary bile acids showed a single peak in each case, confirming the completeness of TLC separation.

Fecal bile acid analysis was carried out on 1-, 2-, or 4-day pools using chromic oxide as a fecal recovery standard (10). Analysis represented a minimum of 21 days collection (range 21–40) and was carried out by the method of Grundy, Ahrens, and Miettinen (14) as previously described.

Three HT subjects in whom [24-^{14}C]cholic and [24-^{14}C]-chenodeoxycholic acid had been administered by vein were studied to determine recovery of ^{14}C-labeled bile acid in stool. Collections were made up to 40 days following administration of radiolabeled bile acid (range 14–40 days). Internal standards of [^{3}H]cholic acid and 3,7-dihydroxy-12-ketocholanic acid were used to correct for procedural losses of radioactivity and mass, respectively, and the overall recovery of acidic steroid was corrected for variation in fecal flow by the use of chromic oxide.

Enzymatic side-chain cleavage

To confirm the distribution of tritium in the side chain of [24,25-^{3}H]cholesterol, each preparation was subjected

to enzymatic cleavage of the C20–C22 bond using the incubation conditions described and validated by Hochberg et al. (11).[2] Briefly, aliquots of a mixture of [24,25-^{3}H]- and [4-^{14}C]cholesterol of known specific activity, were incubated in a medium consisting of a suspension of bovine adrenal mitochondria together with NADPH. This incubation results in cleavage of the C27 sterol into pregnenolone, (C21) and isocaproic acid, (C6). Separation of the product from unreacted substrate was achieved by alumina chromatography. The ^{3}H/^{14}C ratio in cholesterol was determined at the beginning and again, on the unreacted substrate, at the end of the incubation. Isocaproic acid was not retained and appeared in the column void. Cholesterol and pregnenolone were both retained and were eluted separately in methanol and methanol–water 1:1 (v/v), respectively. The two steroid fractions were crystallized to constant specific activity and the pregnenolone fraction was further treated by refluxing in 5% KOH–methanol for 48 hr. After extraction into ethyl acetate and removal of the solvent under nitrogen, the residue was assayed for radioactivity; GLC confirmed the identity of the steroid product as pregnenolone.

Body water kinetics

In seven subjects the size and turnover rate of body water was determined by oral administration of 100 ml of deuterated water (99.5% D_2O abundance, Merck Sharp & Dohme Laboratories, Rahway, NJ). At 3 hr, 24 hr, and subsequent 24-hr intervals up to 10 days, plasma samples were analyzed for atom % excess D_2O. The size and turnover rate constant of body water were then calculated with a single pool model (15, 16). Values for atom % excess D_2O in plasma conformed to a single exponential in every case (data not shown). Analysis of the data was carried out with the SAAM 27 computer program (17).

In addition, body water pool size was indirectly determined in all subjects by means of daily measurements of urinary volume and creatinine excretion (18). Body water pool size estimates did not differ systematically between the two methods.

Statistical analysis

Comparisons were made using Student's *t*-test for paired samples and analysis of variance (ANOVA) as detailed in the BMDP catalog P 1977.

Model development

In all subjects, except for subject #8 (see below), analysis was carried out using the experimental observations of plasma total cholesterol specific activity and ^3H_2O specific activities in both plasma and urine. The latter specific activities were essentially identical and were combined in compartment 18, the body water compartment as shown in the model (Fig. 1). In subjects 1–7, the sizes of compartments 18 and L(0,18) were *fixed* at values obtained from D_2O kinetics. In subject #8, the size of compartment 18 was determined from creatinine excretion and L(0,18) was fixed at 0.11 day^{-1}, the average value from subjects 1–7 (range 0.07–0.15 day^{-1}).

Several assumptions were made in the multicompartmental analysis. First, it was assumed that a steady state was present, both in regard to cholesterol and body water metabolism. Second, the analysis was based on the premise that ^{3}H released into body water was derived solely from biological oxidation of the side chain of [24,25-^{3}H]cholesterol during its conversion into bile acids and steroid hormones. Therefore, possible ^{3}H release from either labile tritium or unreliable [^{3}H]cholesterol was evaluated. To determine the extent to which labile tritium, associated with the injected material, might contribute to the early appearance of ^3H_2O, the following control studies were performed. Aliquots of each preparation were heated to 120°C or incubated at 37°C overnight. Five ml of human plasma was added to each preparation and the mixture was incubated for a further 2 hr at 37°C. Samples were subsequently lyophilized and ^3H_2O radioactivity was determined and expressed as a percent of the starting dose. Preparation #1 exhibited 0.055% (37°C) or 0.085% (120°C) of the dose as free ^3H_2O, while preparation #2 exhibited 0.13% and 0.24% following 37°C and 120°C incubation, respectively. Each value represents the mean of duplicate incubations. The average percent labile ^{3}H in each preparation was therefore incorporated into the model as dose (initial condition) of ^3H_2O in compartment 18. The effect of this labile ^{3}H (injected ^3H_2O) was to reduce the final value for cholesterol oxidation by 1–4% compared to the value when no ^3H_2O dose was injected. It was found that tritium from biologically unreliable [^{3}H]cholesterol (12) did not appear as ^3H_2O. In summary, the second assumption was tested and found to be valid as long as the small amount of labile ^3H_2O in the injected material was incorporated into compartment 18 as dose (initial condition).

In an initial attempt to fit the body ^3H_2O data in compartment 18, plasma total cholesterol was used as the ^3H_2O precursor. A forcing function was used for the total cholesterol data (19). The simulated appearance of ^3H_2O during the initial period (days 1–5) was far below the observed ^3H_2O data. The observed rapid increase in ^3H_2O specific activity could not be generated, even with the labile ^{3}H as initial condition in compartment 18.

The next step was to try plasma free cholesterol and esterified cholesterol, separately, as the precursor of body ^3H_2O data. Since these fractions were individually assayed only in subject #8, it was necessary to derive, mathemati-

[2]These studies were kindly conducted by Dr. Seymour Lieberman, College of Physicians and Surgeons, New York, NY.

cally, the theoretical specific activities of the free and esterified cholesterol in patients 1–7. In addition, since the free and esterified cholesterol specific activities were not direct observations, a forcing function could not be used. Therefore, additional modeling was necessary to generate the shape of the total plasma specific activity–time curve between days 0–21.

The model developed for the above purpose is shown in **Fig. 1**. The observed total cholesterol specific activity is represented by 'summer' compartment 45. This is not a real compartment in the modeling process, but represents the algebraic sum of theoretical specific activities in compartments 44 and 55, plasma free and esterified cholesterol, respectively. The derivation of plasma free and esterified cholesterol specific activities was based on the following. First, in normal and hypertriglyceridemic patients the plasma esterified cholesterol rate constant, L(66,55), is 0.6 day^{-1} (20) and in hypercholesterolemic patients this rate constant is about 0.3 day^{-1} (20–22). L(66,55) was *fixed* accordingly in each analysis. Second, in normal subjects and patients with hyperlipidemias of the types in this study, esterified cholesterol makes up 72% of the total plasma cholesterol, with little variation (20–23). Therefore, to maintain a ratio of plasma esterified to free cholesterol compartment sizes of 2.6 to 1, L(55,44) was *fixed* at 2.6 times the value for L(66,55). Third, previous studies have shown that isotopic cholesterol, when administered in particulate (or collodial) form, is immediately removed from the plasma, to a variable extent, probably by the reticuloendothelial system (19, 24). Therefore, the total dose (initial condition) of ^3H cholesterol was allowed to *vary* between compartments 77 and 44, consonant with the best fit of the total plasma cholesterol specific activity observations. In some studies, such as those shown in Figs. 2A and 2C, the best fit was obtained when the majority of the [^3H]cholesterol dose appeared initially in the plasma. In three studies, such as the one shown in Fig. 2B, the best fit was obtained when most of the [^3H]cholesterol dose was immediately removed from the plasma, by compartment 77, and then gradually reappeared in the plasma cholesterol compartments. Fourth, in order to fit the decay of total [^3H]cholesterol in plasma over the 21-day duration, it was necessary to invoke the presence of two large extraplasma pools, compartments 66 and 77 in Fig. 1. These compartments were each 3 to 10 times larger than the plasma esterified cholesterol mass. The need for two large compartments that exchange with plasma was expected since several investigators have shown that plasma cholesterol decay is bi-exponential when studied for a duration of 3–6 weeks. The configuration of cholesterol exchange between plasma and compartments 66 and 77, as shown in Fig. 1, was not unique and the site of cholesterol input, U(77), was chosen arbitrarily. The magnitude of U(77) was also arbitrary, within limits fixed by literature values (1, 19–23, 25–28). L(66,44),

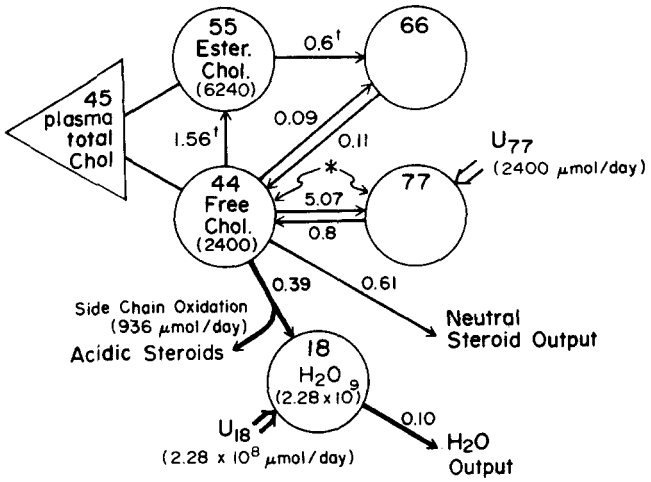

Fig. 1. Multicompartmental model for analysis of [24,25-^3H]cholesterol and body water kinetics. The parameters illustrated are derived from subject #7, a normolipidemic control. Following administration of [24,25-^3H]cholesterol, plasma total cholesterol specific activity was determined daily for 21 days (Methods). Body water (compartment 18), size, and fractional rate constant were determined by use of oral D_2O. Specific activity of free (compartment 44) and esterified (compartment 55) cholesterol were not individually determined but were derived, mathematically, using literature-derived constraints (20–23) as detailed in Methods. Plasma total cholesterol (compartment 45) represents the algebraic sum of specific activity in compartments 44 and 55. To maintain the ratio of plasma esterified to free cholesterol compartments at 72:28 (20–23), L(55,44) was assigned a value 2.6 times that of L(66,55) as indicated by (†). Two additional compartments, 66 and 77, were assigned arbitrary exchange configurations to allow the best fit of the observed biexponential decay of plasma cholesterol specific activity. Tracer input (initial condition) is represented as a bolus (*) between compartments 44 and 77 (24). Input (synthesis plus intake) is represented by U_{77} for cholesterol, and U_{18} for body water, both in μmol/day. Parameters uniquely determined by the model (bold lines) are the rate of cholesterol side-chain oxidation, R(18,44), 936 μmol/day, and the kinetics of body water. Cholesterol removal as neutral steroid, R(0,44) was not quantitated but the sum of rate constants for cholesterol egress (0.61 + 0.39) was forced to equal 1.00. The size of each compartment is shown, in parentheses, in μmol.

L(44,66), L(77,44), and L(44,77) were allowed to *vary* in each patient in order to obtain the best fit of the observed total cholesterol specific activities. In summary, the parameters discussed above in comments 3 and 4 were allowed to vary to fit 'summer' compartment 45; the ^3H_2O data was ignored during this process. L(0,44) was fixed at 1.0 day^{-1} and L(18,44) was fixed at 0.

The above process led to essentially perfect fits of plasma total cholesterol specific activity and to derived curves for free and esterified cholesterol with the constraints imposed by literature values for plasma cholesteryl ester kinetics. All parameters were then fixed; when the derived esterified cholesterol compartment was tested as precursor of body ^3H_2O, the simulated appearance of ^3H_2O during the initial period (days 1–5) was far below the observed ^3H_2O data, as occurred when total cholesterol was used as precursor. When the derived free cholesterol was used as the substrate for ^3H_2O formation, the theoretical curve fit the shape of the ^3H_2O observations in

all studies. L(18,44) was then allowed to vary to obtain the best fit of the amount of ^3H_2O in compartment 18. As L(18,44) varied, L(0,44) was forced to change in a reciprocal fashion so that: L(0,44) + L(18,44) = 1.00. The obtained value for L(18,44), multiplied by the size of compartment 44, represents R(18,44), which is the free cholesterol oxidation rate in μmol per day.

The analysis was simplified in subject #8 in whom direct experimental observations were made of plasma free and esterified cholesterol specific activities. Using a forcing function, the ^3H_2O data was simulated very closely only when plasma free cholesterol was the precursor. Furthermore, in subject #8 the fractional catabolic rate of esterified cholesterol was 0.68 day^{-1} and the esterified to free cholesterol mass ratio was 2.5 to 1; both parameters are very close to the assumptions used in patients 1–7.

The only parameters uniquely determined by the modeling process were the rate of oxidation of plasma free cholesterol and the kinetics of body water (from D_2O). Daily cholesterol input (synthesis plus diet) and loss of cholesterol as neutral sterol could not be determined.

RESULTS

Radiochemical reliability and tritium distribution (in vivo study)

The results of studies performed in six patients are shown in **Table 2**. When compared to the $^3H/^{14}C$ ratio of the administered mixture, the observed $^3H/^{14}C$ ratio in plasma cholesterol fell in five of the six patients. (We have no explanation for the lack of change in patient 14 in the $^3H/^{14}C$ ratio in plasma cholesterol that we have consistently seen in other patients.) Analysis of simultaneously obtained bile and plasma samples for cholesterol specific activity showed (in five of six) a systematic difference in the $^3H/^{14}C$ ratio between the fluids, the greatest discrepancy being found in patient 14. Since, in preliminary experiments, none of the ^3H from biologically unreliable tritiated cholesterol was found in body water and since the absolute *dose* of [24,25-^3H]cholesterol administered to each patient had no effect on the calculation of side chain oxidation rate, determination of the extent of biological unreliability of the [24,25-^3H]cholesterol was not a prerequisite to the kinetic analysis proposed.

Analysis of biliary bile acids showed that those patients infused with preparation #1 had no tritium in either cholic, chenodeoxycholic, or deoxycholic acids and hence it can be assumed that no tritium was located proximal to C24. In contrast, all three patients infused with preparation #2 were discovered to have significant tritium labeling of biliary bile acids—the data further indicating that primary and secondary bile acids were labeled to the same extent in each patient. In calculating the percent tritium located proximal to C24 (Table 2) in each patient, the $^3H/^{14}C$ ratio in each biliary bile acid was divided by the $^3H/^{14}C$ ratio in biliary cholesterol from the same sample. The mean figure, derived by averaging all the observations, for percent tritium located proximal to C_{24} in preparation #2 was 20.7%.

TABLE 2. Radiochemical reliability and tritium distribution of two preparations of [24,25-^3H]cholesterol

Patient	Preparation[a]	Administered Ratio ($^3H/^{14}C$)	Observed Ratio ($^3H/^{14}C$) as Cholesterol[b]		Observed Ratio ($^3H/^{14}C$) as Biliary Bile Acid[c]			% Tritium Located Proximal to C_{24}[d]
			Plasma	Bile	Cholic	Chenodeoxycholic	Deoxycholic	
9	1	2.205	1.914 ± 0.048 (9)	2.266 ± 0.153 (6)				
10	1	2.205	1.938 ± 0.035 (9)	2.194 ± 0.218 (6)	No ^3H counts observed in biliary bile acid			0%
11	1	2.860	2.586 ± 0.090 (9)	2.491 ± 0.113 (5)				
12	2	3.901	2.979 ± 0.073 (8)	3.716 ± 0.185 (4)	0.873 ± 0.009 (4)	0.793 ± 0.061 (4)	0.814 ± 0.095 (4)	23.0 ± 1.2
13	2	3.940	3.115 ± 0.087 (8)	3.636 ± 0.305 (5)	0.841 ± 0.060 (5)	0.861 ± 0.220 (5)	0.927 ± 0.280 (5)	23.7 ± 4.7
14	2	2.220	2.222 ± 0.118 (18)	3.248 ± 0.351 (5)	0.538 ± 0.033 (5)	0.458 ± 0.016 (5)	0.599 ± 0.130 (5)	15.4 ± 1.7

[a] Preparations of [24,25-^3H]cholesterol are as defined in text.

[b] Plasma and biliary cholesterol specific activity ratios were determined at 48-hr intervals over a range of 12 to 20 days (number of analyses in parentheses). Values are mean ± SD.

[c] Bile samples were analyzed for neutral and acidic steroid radioactivity measurements as described in Methods. Acidic fraction was further analyzed by TLC and radioactivity measurements were made on each of the major biliary bile acids. Quantitative separation by TLC was validated by GLC in each case. Values are mean ± SD.

[d] Calculated as $^3H/^{14}C$ ratio in acidic steroid $^3H/^{14}C$ ratio in neutral steroid fraction of same sample. The final value for % ^3H proximal to C_{24} represents the averaged values for each patient's study.

Tritium distribution (in vitro incubation study)

To confirm the observations of tritium distribution (Table 2) in preparation #2, the $^3H/^{14}C$ ratio of the product, pregnenolone, and unreacted substrate, cholesterol, was examined following incubation with bovine adrenal mitochondria. The $^3H/^{14}C$ ratio of recrystallized pregnenolone was 2.83, while that of the remaining cholesterol substrate was 20.7. Since the enzyme specifically cleaves the C20–22 bond, the only explanation for the discovery of ^3H-labeled pregnenolone is the presence of tritium proximal to C_{22}. Results of this in vitro study suggest that 13.7% (2.83 ÷ 20.7) of the tritium in preparation #2 is located proximal to C_{22}. The $^3H/^{14}C$ ratio of the starting material was 20.9, indicating that no substantial changes occurred during the incubation and subsequent isolation procedures.

Upon reflux with methanolic KOH and subsequent reisolation, the $^3H/^{14}C$ ratio of the pregnenolone fell to 0.45, indicating that alkaline saponification had resulted in loss of over 80% of the tritium (at positions proximal to C_{22}) possibly by enolization.

Thus, since earlier work with preparation #2 of [24,25-^{3}H]cholesterol had shown that less than 0.5% of the tritium was located proximal to C_{17} (7), the results of our investigation suggest that approximately 20% of the tritium is *not* at positions 24 and 25, with some 13.7% located at either C_{17} or C_{21} (since the C_{20} is ketonic) and presumably 6% at either C_{22} or C_{23}.[3]

This information is critical to an accurate calculation of the cholesterol oxidation rate using the ^3H_2O data, since approximately one-fifth of the [24,25-^{3}H]cholesterol (preparation #2) is unavailable for liberation into body water upon side-chain oxidation. Specific activity data for those patients who were studied with this preparation (1(b), 6, and 8) were correspondingly adjusted (multiplied by 0.8) to account for this.

Multicompartmental analysis of [24,25-^{3}H]cholesterol and kinetics

Using the model represented by Fig. 1, theoretical plasma free and esterified cholesterol specific activity data were derived from the total plasma cholesterol specific activity values for each patient (**Fig. 2**). When the free (but not the total or esterified) cholesterol specific activity curve was then used as the precursor for ^3H_2O, the simulated ^3H_2O values reproduced the observed conformation of ^3H_2O specific activity with time in each patient without systematic discrepancy. Relevant to this is the fact that the curve fitting for normal and hypertriglyceridemic subjects was no different and that the model was able to predict the shape of the observed ^3H_2O curve over a wide range of daily bile acid synthesis rates, as evidence by the studies before and during cholestyramine therapy in patient #1. No differences were noted between preparation #1 and preparation #2 in terms of the accuracy of the ^3H_2O specific activity curve prediction (Fig. 2, compare panels 2B and 2C).

Bile acid production determined by chemical balance and multicompartmental analysis of [24,25-^{3}H]-cholesterol degradation

Values for bile acid production by chemical balance methodology are listed in **Table 3**. Excluding the two patients studied while taking cholestyramine, there are no differences between fecal bile acid excretion in the six hypertriglyceridemic patients (10.41 ± 2.82 μmol/kg · day) and the normolipidemic subject (10.64 μmol/kg · day). Values for bile acid production in the six hypertriglyceridemic patients were significantly greater ($P < 0.01$) by the [24,25-^{3}H]cholesterol method than by chemical balance methodology when analyzed either as absolute values (mmol/day) or as weight-normalized values (μmol/kg · day) (Table 3). By contrast, one subject with normal plasma lipid values had a value for bile acid production by the [24,25-^{3}H]cholesterol method of 12.37 μmol/kg · day compared to 10.63 μmol/kg · day by chemical balance methodology. Allowing 50 mg (130 μmol) for the daily production of steroid hormones from cholesterol (29, 30), which would result in "non-bile acid" routes of cholesterol oxidation, the methods yield values for bile acid production in this subject of 0.705 versus 0.718 mmol/day by the cholesterol side-chain oxidation and balance methods, respectively. A second normal control subject (#8) had a value for bile acid production of 1.04 mmol/day when similarly corrected for steroid hormone synthesis. These values for bile acid production rates are similar to values determined by Duane et al. (31) using a similar approach following [26-^{14}C]cholesterol administration to three normolipidemic subjects. The values, moreover, are strictly in the range determined by chemical balance methodology in normolipidemic subjects (25–28, 32–36).

Recovery of [24-^{14}C]cholic and [24-^{14}C]chenodeoxycholic label in the stool of hypertriglyceridemic subjects

As an attempt to address the discrepancy between apparent bile acid production and output in hypertriglyceridemic subjects, we examined the possibility of there being a systematic loss of bile acid entering the intestinal lumen in these patients. In three such subjects, the recovery of ^{14}C counts in fecal bile acids was determined after intravenous administration of [24-^{14}C]cholic and

[3]Since the completion of this work, a report has appeared (Rosenfeld, R. S., I. Paul, and B. Zumoff. 1983. *J. Lipid Res.* **24:** 781–783) which confirms these results in a single preparation of [24,25-^{3}H]cholesterol.

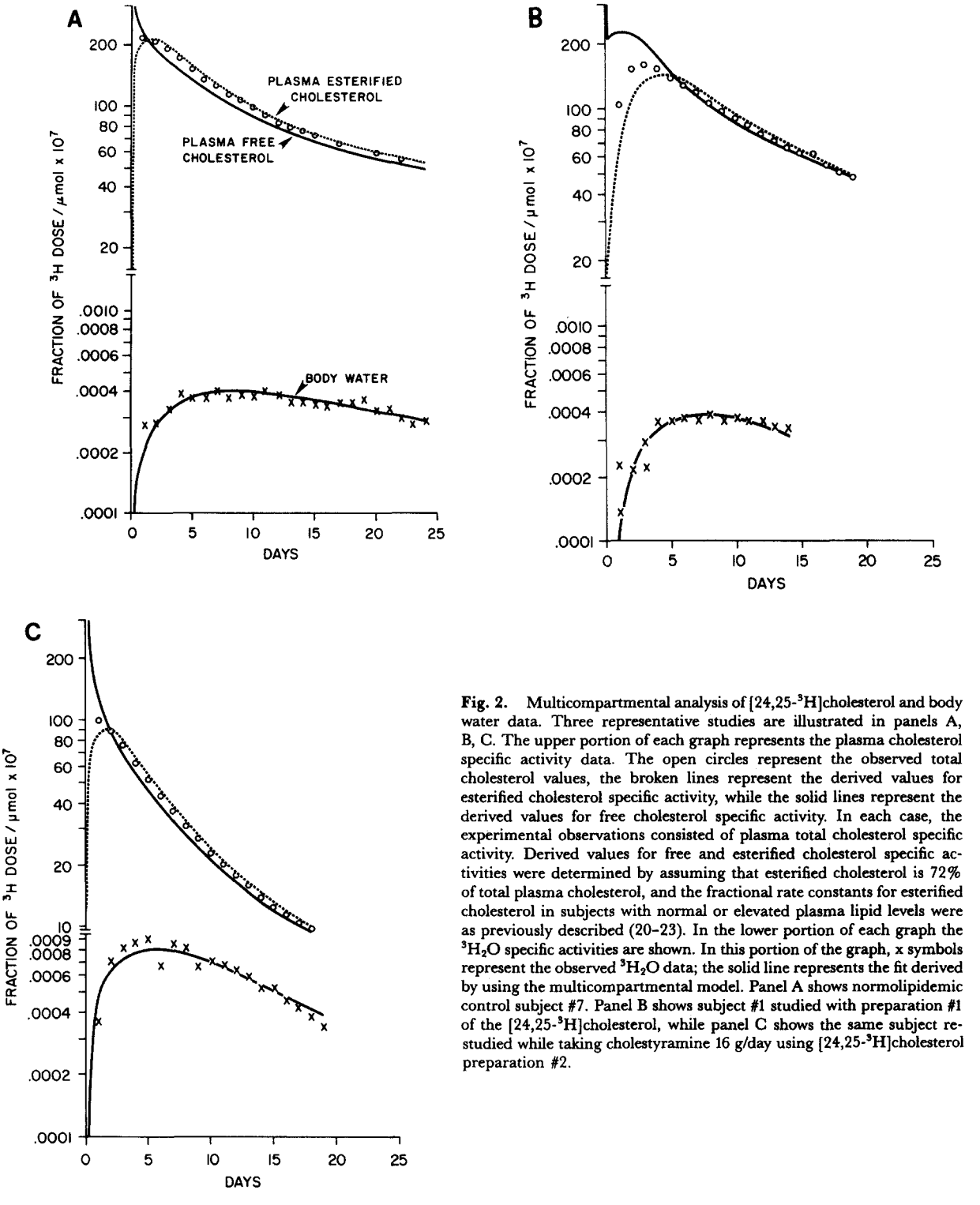

Fig. 2. Multicompartmental analysis of [24,25-^{3}H]cholesterol and body water data. Three representative studies are illustrated in panels A, B, C. The upper portion of each graph represents the plasma cholesterol specific activity data. The open circles represent the observed total cholesterol values, the broken lines represent the derived values for esterified cholesterol specific activity, while the solid lines represent the derived values for free cholesterol specific activity. In each case, the experimental observations consisted of plasma total cholesterol specific activity. Derived values for free and esterified cholesterol specific activities were determined by assuming that esterified cholesterol is 72% of total plasma cholesterol, and the fractional rate constants for esterified cholesterol in subjects with normal or elevated plasma lipid levels were as previously described (20–23). In the lower portion of each graph the ^3H_2O specific activities are shown. In this portion of the graph, x symbols represent the observed ^3H_2O data; the solid line represents the fit derived by using the multicompartmental model. Panel A shows normolipidemic control subject #7. Panel B shows subject #1 studied with preparation #1 of the [24,25-^{3}H]cholesterol, while panel C shows the same subject restudied while taking cholestyramine 16 g/day using [24,25-^{3}H]cholesterol preparation #2.

chenodeoxycholic acids. **Fig. 3** illustrates that in two of the patients, who were studied for up to 42 days, recovery of label was practically quantitative (100 and 93%, respectively). One patient was studied for a shorter time, only 14 days, but recovery of ^{14}C counts in fecal bile acids at this point was similar to values previously reported from this laboratory (14) for normoglyceridemic patients. Thus, these results show that the C_{24} moiety of bile acids is preserved through repeated enterohepatic cycles and quantitatively recovered in the stool as fecal bile acid. Further-

TABLE 3. Daily bile acid synthesis measurements in eight study subjects

Patient	Phenotype[a]	Study[b]	Mean Daily Bile Acid Excretion Measured by Chemical Analysis: Pools Analyzed[c]	mmol/day[d]	μmol/kg · day	Daily Cholesterol Oxidation Rate Measured by [24,25-^{3}H]Cholesterol Kinetics: mmol/day	μmol/kg · day
1	CH	a	12 (2)	0.865 ± 0.138	10.68	1.050 ± 0.032	12.96
		b	6 (4)	4.153 ± 0.873	57.28	5.785 ± 0.116	79.79
2	CH	a	9 (1-4)	0.850 ± 0.190	9.44	2.334 ± 0.058	25.93
		b	5 (4)	5.705 ± 0.490	63.39	9.655 ± 0.097	107.28
3	CH		5 (4)	0.585 ± 0.113	8.13	2.064 ± 0.033	28.67
4	HG		4 (4)	0.923 ± 0.118	13.77	1.214 ± 0.022	18.12
5	HG		5 (4)	0.515 ± 0.155	6.87	1.883 ± 0.049	25.11
6	CH		6 (4)	1.078 ± 0.200	13.55	2.300 ± 0.046	28.93
Mean ± SD	(CH + HG)[e]			0.803 ± 0.213	10.41 ± 2.82	1.808 ± 0.55*	23.29 ± 6.40*
7	NL		7 (2)	0.718 ± 0.178	10.64	0.835 ± 0.013	12.37
8	NL					1.173 ± 0.043	16.16

[a] CH, combined hyperlipidemia; HG, hypertriglyceridemia; NL, normal plasma lipid values.
[b] Study period (a) refers to basal measurements; period (b) refers to study conducted with new steady state during cholestyramine treatment (16 g/day).
[c] Total number of pools analyzed; figures in parentheses indicate 1-, 2-, 3-, or 4-day collections.
[d] Mean ± SD.
[e] Data refer to measurements in patients receiving no medication, i.e., studies 1a, 2a, 3, 4, 5, 6.
* Results compared by paired *t*-test to corresponding fecal bile acid output values show differences to be significant, $P < 0.01$.

more, two of the subjects studied were consuming a solid food diet, suggesting that the discrepancies described above cannot necessarily be ascribed to consumption of liquid formula feeding.

DISCUSSION

Quantitation of cholesterol side chain oxidation as an index of bile acid synthesis has been undertaken in both rabbits (3) and monkeys (4) and the results were found to be comparable to chemical measurement of fecal bile acid output. The use of [24,25-^{3}H]cholesterol to measure bile acid production in man was proposed by Rosenfeld et al. (7). These authors used the incremental change in ^3H_2O specific activity over 12-hr periods up to 96 hr following isotope administration to deduce the rate of cholesterol oxidation. A critical assumption in their calculation was that ^3H_2O lost from the body was insignificant in comparison to the input over any 12-hr period up to 96 hr. We have avoided this potentially major source of error in their method by independently measuring body water kinetics in each subject.

The results illustrate that multicompartmental analysis of the data obtained following [24,25-^{3}H]cholesterol administration provides an accurate simulation of ^3H_2O appearance and turnover over a wide range of bile acid synthesis values. However, as predicted by previous studies, the ^3H_2O data was accurately simulated only when free cholesterol was used as the substrate for oxidation. That the simulated ^3H_2O values reproduced the observed values in all subjects indicates that no conceptual fault exists with the model when applied to studies in hypertriglyceridemic subjects. When steroid hormone synthesis

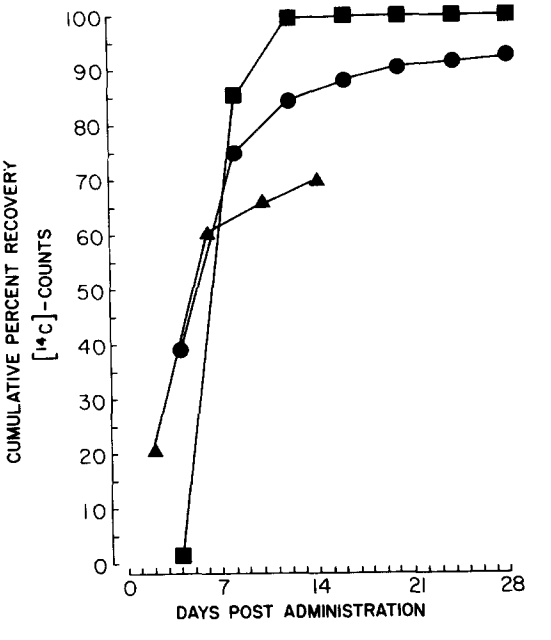

Fig. 3. Cumulative recovery of ^{14}C counts as fecal acidic steroid following intravenous administration of [24-^{14}C]cholic acid and [24-^{14}C]-chenodeoxycholic acid to three hypertriglyceridemic subjects. Stools from each patient were collected for up to 40 days following intravenous coadministration of the [24-^{14}C]-labeled primary bile acids. Following saponification and extraction of the neutral steroids, the acidic steroid fraction was extracted according to the method of Grundy, Ahrens, and Miettinen (14) and the TLC fraction of acidic steroid was subjected to liquid scintillation counting.

from cholesterol (29, 30) was factored into the estimate of cholesterol oxidation, results for bile acid production by the two methods were in close agreement in one normal subject, the discrepancy being 2%. Bile acid production as determined by ^3H_2O formation in the hypertriglyceridemic subjects, however, was still markedly elevated (2.3-times higher, range 1.1–3.4) over bile acid (chemical) excretion rates measured in the same subject.

In determining bile acid production by the [24,25-^{3}H]-cholesterol oxidation method, a major assumption is that tritium released into body water reflects, exclusively, oxidation of the side chain of [24,25-^{3}H]cholesterol during its conversion to bile acids and steroid hormones. Several characteristics of the isotopically labeled cholesterol were therefore investigated. Minor lability of tritium (0.05–0.24%) was demonstrated following incubation of each radiolabeled cholesterol preparation. This observation allowed for an initial dose of ^3H_2O into the water compartment to account for a small portion of the early appearance of ^3H_2O. However, its impact upon apparent bile acid production was small (<4%). Of further importance was the observation that none of the tritium lost from plasma [^{3}H]cholesterol in the initial period of removal of the biologically unreliable material was found as ^3H_2O. Furthermore, there was no change in plasma cholesterol $^3H/^{14}C$ ratio after the initial fall (Table 2) from the administered $^3H/^{14}C$ ratio, suggesting that additional ^3H lability did not occur in vivo. Recirculation of ^3H_2O back into newly synthesized cholesterol was negligible; the specific activity of ^3H_2O was a factor of 10^6 lower than that of tritiated cholesterol in all subjects. The results of two independent investigations into tritium distribution in [24,25-^{3}H]cholesterol revealed that, in preparation #2, approximately 20% of the ^3H label was located proximal to C_{24}, with approximately 13.7% proximal to C_{22}. In preparation #1, by contrast, no ^3H was located proximal to C_{24}. The presence of "ectopic" tritium in preparation #2, used in patients 1(b), 6, and 8, necessitated an adjustment in the plasma cholesterol specific activity values since only 80% of the [24,25-^{3}H]cholesterol was actually available for tritium liberation into the body water upon cholesterol side-chain oxidation. The qualitative appearance of the ^3H_2O specific activity data and the values for oxidation of cholesterol were not systematically different in studies using the different preparations of [24,25-^{3}H]-cholesterol (Table 3 and Fig. 2). With both preparations the simulated ^3H_2O specific activity data were representative of the observed values. With both preparations in patient #1, bile acid synthesis, as measured by cholesterol side-chain oxidation, exceeded bile acid chemical excretion by factors of 1.23 and 1.39, respectively. Thus, over a wide range of absolute bile acid synthesis, the evidence from this subject indicates that both preparations of [24,25-^{3}H]cholesterol give values with a similar relationship to bile acid excretion as measured by chemical balance methodology. Provided investigators are aware of these potential hazards, the use of either preparation of [24,25-^{3}H]cholesterol in human studies, as described, should provide meaningful information.

A major conclusion of this study is that the specific activity of plasma free cholesterol alone reflects the kinetics of the precursor for hepatic bile acid synthesis. This hypothesis was tested and validated directly in a control subject (#8) and confirms previous studies in which isotopically labeled precursors were used to determine the sources of substrate for hepatic bile acid synthesis (19, 37). In addition, the effect of varying the FRC for plasma esterified cholesterol was tested directly in order to observe the outcome on bile acid production by the cholesterol side chain oxidation method. In changing the FRC from values of 0.2/day up to 1.0/day, values for bile acid production changed by less than 15% indicating that, within limits, the assumptions used in deriving the values for plasma free cholesterol specific activity were not themselves potent determinants of the final value for cholesterol side-chain oxidation rate. Furthermore, since the assumptions were the same for the control and hypertriglyceridemic patients, we conclude that the results illustrate a real difference in bile acid production in the latter patients.

Four hypertriglyceridemic patients (#1a, 2a, 3, 4) previously studied by isotope dilution kinetics of [^{14}C]cholic and [^{14}C]chenodeoxycholic acids (13), were involved in the present study protocol. Their clinical and dietary features were unchanged and the steady state was not perturbed. The data shown in **Fig. 4** illustrate the results of measuring bile acid production by all three independent methods. In three of four patients bile acid synthesis values, as measured by the kinetics of cholesterol oxida-

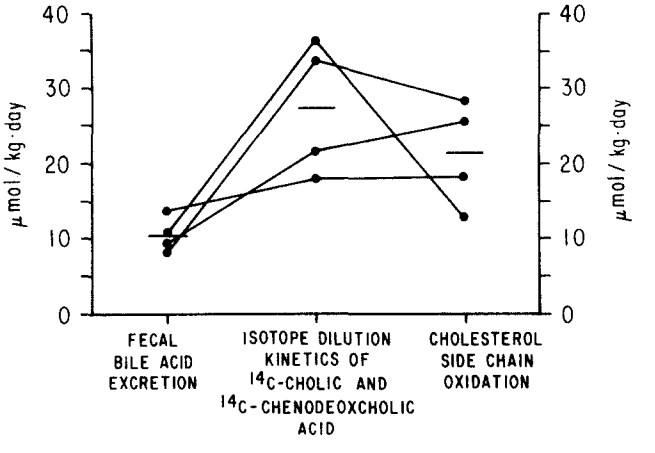

Fig. 4. Measurement of bile acid production in four hypertriglyceridemic subjects (#1a, 2a, 3, 4) by three independent methods. The subjects were previously studied using isotopic bile acid dilution kinetics (13) and were subsequently studied by the cholesterol side-chain oxidation method. The mean for the group by each method is indicated by the horizontal bar. Differences, determined by paired analysis, were found to be significant between chemical balance and isotope dilution kinetics ($P < 0.025$) and chemical balance and [24,25-^{3}H]cholesterol oxidation ($P < 0.05$).

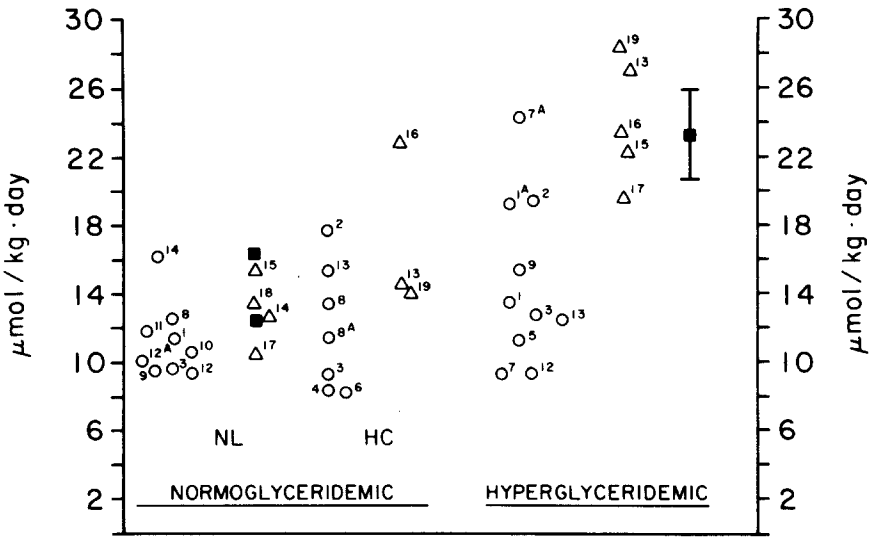

Fig. 5. Bile acid production in normal and hyperlipidemic subjects. A summary of 19 studies quantitating bile acid production in human subjects is presented. Fecal bile acid (chemical) excretion (○) was measured by the technique of Grundy, Ahrens, and Miettinen (14), with few modifications, except where indicated. Seven studies using isotope dilution kinetics (△) of ^{14}C-labeled cholic and chenodeoxycholic acid are presented (38). Studies involving the use of randomly or specifically tritium-labeled bile acids have been excluded from this comparison (13). Bile acid production as determined by the cholesterol side-chain oxidation method (■), in the present study, is also illustrated with values for HT subjects expressed as mean ± SEM. Individual values together with the group mean and standard deviation (23.29 ± 6.40 μmol/kg · day) are presented in Table 3.

Appendix to Fig. 5

Figure Reference	Corresponding Manuscript Reference	Comments
1, 1A	Biel et al. (32)	1A represents subjects with familial combined hyperlipidemia illustrated in comparison to those with poorly classified hyperglyceridemia.
2	Keşäniemi and Grundy (39)	Four HT subjects excreted 19.4 ± 8.8 μmol/kg · day compared to 17.8 ± 3.0 μmol/kg · day for seven hypercholesterolemic (HC) subjects, $P > 0.05$.
3	Grundy et al. (33)	
4	Grundy and Ahrens (40)	
5	Grundy and Ahrens (41)	
6	Shepherd et al. (25)	
7, 7A	Grundy (42)	7A, two subjects with type V hyperlipidemia (50); 7, six subjects with type IV hyperlipidemia (50).
8	Bilheimer, Stone, and Grundy (26)	
9	Nestel and Hunter (27)	Obese HT subjects excreted more bile acid than obese NL subjects.
10	Connor et al. (28)	Data from studies during cocoa butter ingestion (mean 10.9 ± 4.7 μmol/kg · day); no Cr_2O_3 used.
11	Nestel, Schreibman, and Ahrens (34)	Obese NL subjects.
12	Miettinen (35)	No differences in bile acid excretion between NL and HT subjects.
Studies 13–19 used isotope dilution kinetics of [^{14}C]cholic and [^{14}C]chenodeoxycholic acids (38).		
13	Davidson et al. (13)	No differences in bile acid excretion between HC and HT subjects. HT subjects produce more bile acid than HC subjects.
14	Duane and Hutton (36)	No difference between bile acid input (38) and excretion in NL subjects.
15	Einarsson, Hellström, and Leijd (43)	Type IV subjects (50) produce more bile acid than type IIA subjects (50).
16	Nilsell et al. (44)	No difference between HT and HC subjects. Values for HC subjects (22.9 ± 12.3 μmol/kg · day, n = 5) higher than previously reported by same investigators (43, 45).
17	Angelin et al. (45)	Type IV subjects (47) produce more bile acid than type IIA subjects (50).
18	Leijd (46)	Non-obese subjects.
19	Leijd (47)	Obese HT subjects compared to same subjects following weight reduction and normalization of plasma TG levels.

tion or dilution of primary bile acids (38), were within 10% of one another. The fourth patient appeared anomalous in this regard. Values for fecal bile acid (chemical) excretion, by contrast, were significantly lower than the corresponding values obtained by either kinetic analysis (Fig. 4). In this small collection of hypertriglyceridemic subjects, therefore, kinetic determinations were found to demonstrate reasonable concordance with one another and to be different from fecal bile acid chemical excretion. This observation needs further corroboration before it can be hypothesized that there exists a real difference between the various methodologies in hypertriglyceridemic subjects.

Bile acid production in human subjects has been the focus of many studies. A representative, but not exhaustive, collection of such data is presented in **Fig. 5** (13, 25–28, 32–36, 39–47). It is evident that, when represented by fecal bile acid excretion, values for bile acid production are in the range of 8–16 μmol/kg · day, with the majority of studies showing no systematic discrepancy attributable to hyperlipidemic phenotype. There may be a subset of hypertriglyceridemic patients (studies 1A, 7A, Fig. 5) who indeed excrete more bile acid than normal subjects but the majority of such patients do not. By contrast, studies examining bile acid synthesis by isotope dilution kinetics demonstrate an unequivocal trend to overproduction in hypertriglyceridemic subjects (Fig. 5). The values obtained in the present study are included for reference and appear to be within the range reported for subjects appropriately matched by phenotype. Other investigators (48) using different methodology have reported that hypertriglyceridemic subjects degrade more cholesterol than normoglyceridemic controls. Furthermore, although not addressed in the present study, cholesterol elimination as neutral steroid may also vary with hyperlipidemic phenotype (42).

Evidence suggests that the C_{24} moiety of acidic steroids is conserved during passage through the intestine of three hypertriglyceridemic subjects, but the question of intralumenal degradation cannot presently be answered. The concordance of isotopic methods in these subjects (Fig. 4) may, in part, reflect their relative independence from the effects of possible colonic bile acid degradation. The precedent for steroid metabolism by intestinal bacteria in humans (49) deserves further attention, particularly with reference to different hyperlipidemic populations.

Further studies on the use of [24,25-^{3}H]cholesterol complexed to various lipoprotein fractions are currently being conducted to elucidate details of hepatic bile acid metabolism in various dyslipoproteinemic states.

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