

# Multicompartmental Analysis of Cholesterol Metabolism in Man

## QUANTITATIVE KINETIC EVALUATION OF PRECURSOR SOURCES AND TURNOVER OF HIGH DENSITY LIPOPROTEIN CHOLESTEROL ESTERS

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**ABSTRACT** The purpose of this study is to delineate the immediate sources and fractional turnover of high density lipoprotein (HDL) esterified cholesterol in man. Various labeled preparations were administered in 11 experiments to six subjects who had either a complete bile fistula (maximally stimulated cholesterol metabolism) or an intact enterohepatic circulation. The administered tracers included [ $^3\text{H}$ ]mevalonic acid; [ $^{14}\text{C}$ ]cholesterol bound to albumin; low density lipoprotein (LDL) free [ $^3\text{H}$ ] or [ $^{14}\text{C}$ ]cholesterol; HDL free [ $^3\text{H}$ ] or [ $^{14}\text{C}$ ]cholesterol; HDL esterified [ $^3\text{H}$ ]cholesterol; and LDL esterified [ $^3\text{H}$ ]cholesterol. Blood samples were obtained at frequent intervals for up to 5 d after the administration of tracers. The mass and radioactivity in individual plasma lipoprotein (very low density lipoprotein [VLDL], HDL, and LDL) free and esterified cholesterol were determined.

The data were subjected to multicompartmental analysis using the SAAM-27 computer program. The analysis revealed that plasma free cholesterol was not the only immediate source of either a single- or two-compartment HDL ester system. When LDL esters and plasma (HDL) free cholesterol were tested together as sources of one HDL ester compartment, data from all the experiments were readily fit.

The fluxes arrived at with the final model indicated that only ~20% of the esterified cholesterol in HDL was newly synthesized from plasma (HDL) free cholesterol (2.36  $\mu\text{mol}/\text{min}$ ); the remaining 80% was from LDL ester (8.92  $\mu\text{mol}/\text{min}$ ). The presence of a bile

fistula had no obvious effect on HDL esterified cholesterol metabolism. The rate of HDL cholesterol ester turnover was 3–12 times/d, indicating that the ester component of the HDL particle is in a very dynamic state.

### INTRODUCTION

According to present concepts, the plasma lecithin-cholesterol acyltransferase, in concert with cholesterol ester transfer proteins, form the primary system responsible for the synthesis and intravascular transport of cholesterol esters in man. The lipoprotein site of origin of the esters formed by lecithin-cholesterol acyltransferase appears to be high density lipoprotein (HDL<sub>3</sub>)<sup>1</sup>, which has been shown to be the preferred substrate for the enzyme (1). In vitro studies with human plasma suggest that the newly synthesized esters are then coupled to the cholesterol ester exchange protein and transferred to VLDL and LDL (2, 3). Delipidation of VLDL by lipoprotein lipase action leads to the formation of intermediate density lipoprotein (IDL) and then LDL (4). Completion of the cholesterol

<sup>1</sup> *Abbreviations used in this paper:* ACAT, acyl-coenzyme A cholesterol acyltransferase (EC 2.3.1.26); C(9), HDL esterified cholesterol compartment; HDL, high density lipoprotein; LCAT, lecithin-cholesterol acyltransferase (E.C. 2.3.1.43); LDL, low density lipoprotein;  $R(m, n)$ , mass of material in compartment  $n$  transported to compartment  $m$  per minute; VLDL, very low density lipoprotein;  $\alpha$ , lipoproteins (HDL) in the supernate after treatment of plasma with heparin-manganese;  $\beta$ , lipoproteins (LDL plus VLDL) in the precipitate after treatment of plasma with heparin-manganese.

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ester cycle is thought to occur via the lysosomal hydrolysis of LDL cholesterol esters initiated by the cellular LDL receptor systems (5). Although this has been a reasonable working hypothesis to describe plasma lipoprotein cholesterol ester metabolism, additional complexities are present since plasma transfer proteins have also been shown *in vitro* to promote the bidirectional exchange of cholesterol esters between HDL and LDL and between HDL and VLDL (6). Although recent studies in man (7) with injected labeled HDL and LDL cholesterol esters tend to substantiate the presence of these cholesterol ester exchange reactions *in vivo*, the function of these exchange reactions and their quantitative significance *in vivo* have not been elucidated.

Previous investigations on the *in vivo* metabolism of cholesterol esters in man have involved the administration of labeled mevalonic acid and free cholesterol, and subsequent kinetic analysis of the HDL, VLDL, and LDL cholesterol ester specific activity curves (8–10). It has been observed that HDL esters become most rapidly labeled, followed by the VLDL and then LDL esters. From these types of data it has been inferred that HDL esters are newly synthesized from free cholesterol, HDL esters turn over rapidly, and HDL represents the major source of cholesterol esters in the other lipoproteins. However, neither these possibilities nor the *in vitro* findings have been quantitated or substantiated *in vivo*. The inherent difficulties of interpreting the complex lipoprotein cholesterol ester specific activity decay curves, which are a composite of *de novo* synthesis and exchange reactions, has made it difficult to obtain valid information on HDL cholesterol ester turnover and synthesis in man.

In the present study these methodologic problems have been resolved in order to determine HDL ester turnover and the sources of HDL cholesterol esters *in vivo*. Subjects were injected with a variety of labeled preparations (mevalonic acid, lipoprotein free cholesterol, LDL cholesterol ester, HDL cholesterol ester). The problem caused by free [ $^3\text{H}$ ]cholesterol in the LDL- and HDL-esterified [ $^3\text{H}$ ]cholesterol preparations has been circumvented by the inclusion of free [ $^{14}\text{C}$ ]cholesterol in the injected preparations. By simultaneously labeling different lipoprotein cholesterol compartments, and then performing multicompartmental kinetic analysis, we have derived a working model that both quantitatively and qualitatively describes HDL ester metabolism in man.

## METHODS

**Patient description.** Information on the six subjects used in the experiments is shown in Table I. Informed consent was obtained from each subject and the study was approved by the Committee for the Conduct of Human Research. Sub-

jects 1, 2, 4, and 6A had a complete bile fistula at the time of the experiments. The advantages of using a bile fistula patient to study cholesterol metabolism and its particular relevance for the development of a kinetic model in man have been discussed earlier (11). All patients were ambulatory and consumed a regular hospital diet before the experiments. Their liver function and renal function tests were normal and none was diabetic. The T-tube (bile fistula patients) was allowed to remain open and drain by gravity for 5–7 d before the study; bile was quantitatively collected both before and during the experiments. Bile acid synthesis and bile cholesterol secretion rates reached a steady maximal level after 3–4 d of drainage and the stools were acholic during the experiments.

The labeled compounds administered to each patient are shown in Table II. The duration of each experiment and the interval between the experiments are shown in Table I. All subjects fasted overnight and remained fasting for the first 12 h of the experiment. Subjects 1A, 2A, and 3 received autologous HDL and LDL labeled with  $^3\text{H}$ - and  $^{14}\text{C}$ -free cholesterol, respectively. In the second experiment patients 1B and 2B received HDL and LDL labeled with  $^{14}\text{C}$ - and  $^3\text{H}$ -free cholesterol, respectively. Subjects 4A and 5A received HDL labeled with [ $^3\text{H}$ ]cholesterol ester and dual-labeled free cholesterol ( $^3\text{H}$  and  $^{14}\text{C}$ ). Subject 4B then received similarly labeled LDL in the second experiment. Subjects 5B and 6 were administered [ $^3\text{H}$ ]mevalonic acid with or without  $^{14}\text{C}$ -free cholesterol bound to albumin.

**Experimental design.** The interpretation of radioactivity data obtained after the administration of lipoproteins containing single  $^3\text{H}$  labeled esterified and free cholesterol, has been hampered because of (a) the formation of labeled esters from the injected free precursor and (b) the labeled free cholesterol liberated by hydrolysis of the injected ester. To circumvent these difficulties the free cholesterol fraction of HDL and LDL was also labeled with another free cholesterol tracer, [ $4\text{-}^{14}\text{C}$ ]cholesterol. The appearance (and disappearance) of  $^{14}\text{C}$  activity in the esterified cholesterol fractions allowed for a precise evaluation of the contribution of the injected  $^3\text{H}$ -free cholesterol to the esterified fractions. The ability to trace the metabolism of the injected free [ $^3\text{H}$ ]cholesterol afforded a means of differentiating between lipoprotein cholesterol esters derived from the free precursors and those from the injected lipoprotein cholesterol esters.

Another experimental approach used in several experiments in the present study involved the simultaneous administration of different labeled precursors of the HDL cholesterol esters, namely HDL and LDL free cholesterol, albumin bound [ $^{14}\text{C}$ ]cholesterol, and mevalonic acid. This has allowed for the labeling of the putative precursor free cholesterol compartment by directly administering labeled free cholesterol and also by endogenous synthesis from mevalonic acid. HDL cholesterol esters derived from these unesterified precursors could potentially originate in whole or in part from tissue sources (ACAT) and/or via the plasma lecithin-cholesterol acyltransferase (LCAT) reaction. Rigid constraints were placed on the development of the HDL cholesterol ester model since the same kinetic parameters were required to fit the  $^3\text{H}$  and  $^{14}\text{C}$  ester data obtained after the simultaneous administration of the two precursors. The administration of labeled HDL and LDL cholesterol esters permitted an independent evaluation of HDL cholesterol ester kinetics, which was also constrained by the requirement that  $^3\text{H}$  and  $^{14}\text{C}$  data fit the same model parameters. This multiple-label approach afforded an opportunity to construct an HDL ester model based on the exchange of tracers between compartments rather than on the number of ex-

TABLE I  
Information on Subjects

Subject	Sex	Age	Weight	Height	Status of enterohepatic circuit	Duration of study	Time after administration of previous labels	Esterified cholesterol concentration		Comments§
								HDL*	LDL†	
		yr	kg	in			$\mu\text{mol/dl}$			
1 <sup>  </sup>	M	59	74.5	71	Bile fistula¶	A, 3.0	—	77.3±1.8 (15)**	129.3±2.9 (16)**	No medications
						B, 0.9	3 d	77.6±1.8 (9)	107.0±1.6 (9)	
2	F	68	62.0	65.5	Bile fistula¶	A, 3.0	—	57.4±1.3 (15)	96.4±2.1 (16)	No medications
						B, 0.9	3 d	58.7±1.1 (10)	104.0±3.2 (10)	
3 <sup>  </sup>	M	60	77.7	68.5	Intact	0.4	—	94.2±1.3 (11)	188.2±3.1 (11)	No medications. Triglycerides 76 mgm/dl
4	F	33	105.0	62	Bile fistula¶	A, 1.1	—	67.6±3.0 (11)	117.6±3.1 (11)	No medications
						B, 0.3	1.1 d	64.6±2.2 (5)	127.5±4.1 (4)	
5A <sup>  </sup>	M	86	58	65	Intact	3.9	16 mo	67.7±1.4 (24)	194.7±3.2 (24)	Digoxin, mestrion
5B <sup>  </sup>	M	84	56.4	66	Intact	4.0	—	88.1±1.2 (22)	216.4±7.0 (22)	Digoxin
6A	M	49	86.8	73	Bile fistula††	6.0	—	41.2±1.0 (17)	110.2±2.4 (18)	Alcoholic pancreatitis 5 wk before study
6B <sup>  </sup>	M	49	86.8	73	Intact§§	3.8	3 mo	54.7±1.3 (19)	156.8±3.3 (20)	Chylomicrons occasionally present and removed with VLDL

\* Density 1.063–1.210.

† Density 1.019–1.063, except in 6A and 6B, where LDL represents  $1.006 < d < 1.063$ .

§ All subjects fasted for 16 h before the administration of labels and then for the subsequent 12 h (6 h for subject 6B). No chylomicrons were apparent in any plasma sample after ultracentrifugation except for occasional samples from 6B after the initial 6 h of this experiment. Blood samples obtained following the initial fasting period were taken at least 3 h after meals (usually after an overnight fast).

<sup>||</sup> Out-patient volunteer studied in the Clinical Research Center.

¶ Surgery for cholelithiasis 2–3 wk before the experiments.

\*\* Mean±SE of mean (number of observations).

†† Surgery for common bile duct stricture 3 wk before the experiment.

§§ Bile fistula closed 3 mo before starting experiment B.

potentials in the specific activity time curves. The kinetic model was developed by subjecting the specific activity data to simulation analysis and modeling using the SAAM-27 computer program (12).

**Labeled compounds.** The DL-[5-<sup>3</sup>H]mevalonic acid (DBED salt), [1,2-<sup>3</sup>H]cholesterol, and [4-<sup>14</sup>C]cholesterol were obtained from New England Nuclear, Boston, MA, and stored at -15°C in ethanol. The radiolabeled cholesterol was checked for purity (and purified if necessary) by silicic acid column chromatography, thin-layer chromatography on silica gel G with a solvent system of petroleum ether/diethyl ether/acetic acid (89:11:3, vol/vol/vol), and recovery of radioactivity as cholesterol digitonide. The mevalonic acid was liberated from the DBED salt by the addition of sodium bicarbonate and the DBED was extracted with diethyl ether. The aqueous solution of labeled sodium mevalonate was neutralized with an equimolar amount of HCl, diluted to a volume of 25 ml with sterile saline, passed through a 0.22- $\mu\text{m}$  filter, and administered to the subject. Subject 6 (experiments A and B, Table II) received [4-<sup>14</sup>C]cholesterol with an albumin carrier. The [4-<sup>14</sup>C]cholesterol was dissolved in 0.25 ml of ethanol and slowly added to 20 ml of human serum albumin. The use of particulate radiolabeled cholesterol administered with albumin has been previously discussed (11, 13).

**Labeled lipoproteins.** Fasting blood was obtained from subjects 1–4 and 5A ~3 d before the start of the experiments. The erythrocytes were immediately separated from the plasma by centrifugation at 2,500 rpm at 4°C for 10 min. Several procedures were used to label the lipoproteins with free and esterified cholesterol. Labeling of HDL and LDL with either free [1,2-<sup>3</sup>H]cholesterol or free [4-<sup>14</sup>C]cholesterol in subjects 1A, 1B, 2A, and 2B was carried out as described earlier (14). Plasma was incubated at 4°C with 5-mm discs of Whatman No. 1 filter paper (Whatman, Inc., Clifton, NJ) impregnated with the labeled free cholesterol with gentle agitation for 3 h. The LDL and HDL were then isolated by preparative ultracentrifugation at 4°C. This gave four labeled lipoprotein preparations [<sup>14</sup>C-HDL, <sup>14</sup>C-LDL, <sup>3</sup>H-HDL, and <sup>3</sup>H-LDL]. The individual preparations were dialyzed overnight against saline 4°C, with 0.01% EDTA at pH 7.4 to remove KBr, passed through 0.22- $\mu\text{m}$  filters, and administered to the subjects. The free [<sup>14</sup>C]cholesterol-LDL administered to subject 3 was also prepared in the above manner. The free [<sup>3</sup>H]cholesterol-HDL given to subject 3 was prepared as above, except that the [<sup>3</sup>H]cholesterol labeled whole plasma was first treated with heparin-MnCl<sub>2</sub> (15) followed by removal of the heparin and manganese from the supernate, as described previously (7). This was done to remove all traces of apoprotein-B containing lipoproteins such

TABLE II  
Labeled Compounds Administered to Subjects

Subject and experiment No.	Labeled compounds	Vehicle
1A	40 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	HDL
	1.24 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	LDL
1B	0.32 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	HDL
	50.5 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	LDL
2A	33.5 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	HDL
	0.90 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	LDL
2B	0.63 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	HDL
	25 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	LDL
3	31.2 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	HDL
	1.7 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	LDL
4A	8.14 × 10 <sup>6</sup> dpm esterified [1,2- <sup>3</sup> H]cholesterol	HDL
	18.13 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	
	1.45 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	
4B	2.68 × 10 <sup>6</sup> dpm esterified [1,2- <sup>3</sup> H]cholesterol	LDL
	37.7 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	
	1.04 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	
5A	4.75 × 10 <sup>6</sup> dpm esterified [1,2- <sup>3</sup> H]cholesterol	HDL
	10.50 × 10 <sup>6</sup> dpm free [1,2- <sup>3</sup> H]cholesterol	
	2.40 × 10 <sup>6</sup> dpm free [4- <sup>14</sup> C]cholesterol	
5B	317 × 10 <sup>6</sup> dpm DL [5- <sup>3</sup> H]mevalonic acid	Saline
6A	477 × 10 <sup>6</sup> dpm DL [5- <sup>3</sup> H]mevalonic acid	Saline
	35.5 × 10 <sup>6</sup> dpm [4- <sup>14</sup> C]cholesterol	Albumin
6B	261 × 10 <sup>6</sup> dpm DL [5- <sup>3</sup> H]mevalonic acid	Saline
	31 × 10 <sup>6</sup> dpm [4- <sup>14</sup> C]cholesterol	Albumin

as Lp(a). The <sup>3</sup>H-HDL was then isolated from the remainder of the supernate by ultracentrifugation. Analysis of the lipoprotein preparations in subjects 1–3 showed that (a) >99% of the cholesterol label was unesterified; (b) the label migrated with the appropriate lipoprotein band on agarose gel electrophoresis and the electrophoretic mobility of the HDL and LDL were unchanged (16), and (c) the label appeared in the appropriate hydrated density fraction upon repeat ultracentrifugation.

Labeling of the lipoproteins with free and esterified cholesterol (subjects 4A, 4B, and 5A) was carried out by several procedures as described previously (7). The method for subject 5A was as follows: plasma was incubated with 5-mm discs of Whatman No. 1 filter paper impregnated with [1,2-<sup>3</sup>H]cholesterol under N<sub>2</sub> for 4 h at 37°C. The plasma was decanted from the filter paper, cooled to 4°C, and placed in another vial containing filter paper discs impregnated with [4-<sup>14</sup>C]cholesterol. Incubation was then carried out for an additional 4 h under N<sub>2</sub> at 4°C. The plasma was then decanted and the β-lipoproteins (LDL plus VLDL) were precipitated with heparin and MnCl<sub>2</sub> (15) and discarded. The manganese and heparin were removed, and the supernatant plasma containing HDL was adjusted to 1.21 with solid KBr and subjected to ultracentrifugation for 22 h at 4°C. The HDL fraction was isolated, dialyzed overnight

against saline 4°C with 0.01% EDTA (pH 7.4), passed through a 0.22-μm filter, and administered to the patient.

The procedure for labeling the plasma lipoproteins of subjects 4A and 4B was similar to subject 5A, except that the lipoproteins were isolated exclusively by ultracentrifugation. After the incubation procedures, the plasma was adjusted to *d* 1.063 with KBr and the solution ultracentrifuged for 22 h. The supernatant solution containing VLDL, IDL, and LDL was removed and the density adjusted to 1.019 with water. The solution was centrifuged, the 1.019 supernate was discarded, and the infranate (LDL) was retained. The infranate from the original 1.063 spin was adjusted to *d* 1.21 with solid KBr and the HDL isolated after ultracentrifugation for 22 h. Both HDL and LDL fractions were dialyzed overnight against saline 4°C with 0.01% EDTA (pH 7.4), passed through a 0.22-μm filter and administered to patient 4 in two separate experiments (A and B). Incubation of the plasma with [1,2-<sup>3</sup>H]cholesterol at 37°C for 4 h allowed for the initial formation of labeled HDL-cholesterol ester by the catalytic action of LCAT. Approximately 25–35% of the labeled HDL cholesterol was esterified. The LDL ester fraction also became labeled. This probably occurred via the recently characterized plasma cholesterol ester exchange protein, which has been shown to transfer HDL ester to LDL (2). Subsequent incubation of the plasma at 4°C with [4-

$^{14}\text{C}$ ]cholesterol achieved labeling of only the free cholesterol. The final lipoprotein preparations (HDL and LDL) contained free [ $1,2\text{-}^3\text{H}$ ]cholesterol, free [ $4\text{-}^{14}\text{C}$ ]cholesterol and esterified [ $1,2\text{-}^3\text{H}$ ]cholesterol.

The lipoprotein preparations containing esterified [ $^3\text{H}$ ]cholesterol (subjects 4 and 5A) were checked for homogeneity of mass and label and physiologic activity in the following manner: the lipoproteins (HDL and LDL) each migrated as a single band on agarose gel electrophoresis and paralleled the appropriate known lipoprotein standards. Over 90% of the radioactivity on the agarose plate was associated with the appropriate (HDL or LDL) lipoprotein fraction. Immunoelectrophoresis gave the expected lines of identity with HDL and LDL. When the lipoprotein preparation was ultracentrifuged, the radioactivity floated in the appropriate density range. The  $^{14}\text{C}$  activity was virtually (>99%) all in the free cholesterol fraction, as judged by silicic acid column chromatography, thin-layer chromatography, and digitonin precipitation. Physiologic behavior of the injected lipoprotein preparations in man was assessed on the basis of (a) exponential decay for  $^3\text{H}$ - and  $^{14}\text{C}$ -free cholesterol and [ $^3\text{H}$ ]cholesterol ester, (b) virtually complete recovery of the administered labeled free and esterified cholesterol in the blood compartment within 10 min of injection, and (c) no sudden spike of radioactivity in the bile (7).

**Other procedures.** Blood samples for ultracentrifugation (17) were collected in EDTA (1 mg/ml). For rapid precipitation of  $\beta$ -lipoproteins (15), blood was collected in heparinized tubes (20 USP U/ml). After venipuncture, plasma was immediately separated from erythrocytes by centrifugation for 7 min at  $4^\circ\text{C}$  and 5,5'-dithiobis-(2-nitrobenzoic acid) added to samples for ultracentrifugation to a concentration of 0.02 M. Lipoproteins were separated by successive ultracentrifugation at  $4^\circ\text{C}$  into VLDL ( $\leq 1.006$ ), LDL (1.019–1.063), and HDL (1.063–1.21). Complete separation of the plasma into  $\alpha$ - and  $\beta$ -fractions by the rapid precipitation method was achieved within 15 min of venipuncture, and was confirmed by agarose gel electrophoresis (16).

The lipoprotein fractions were extracted with 20 vol of chloroform/methanol (2:1, vol/vol) and washed with 1/5 vol of water (18). The free cholesterol and esterified cholesterol in the chloroform phase were separated by silicic acid column chromatography (19). The free cholesterol mass was determined by gas-liquid chromatography. The stationary phase was 3% SP 2401 (Supelco, Inc., Bellefonte, PA) and the internal standard was coprostanol. Another aliquot of the free cholesterol fraction was precipitated as the digitonide, dissolved in methanol, and assayed for  $^3\text{H}$  and  $^{14}\text{C}$  activity by liquid scintillation counting. The esterified cholesterol fraction was hydrolyzed with 5% KOH for 1 h, neutralized with HCl, and the liberated free cholesterol precipitated as the digitonide; mass was determined colorimetrically on an aliquot of the digitonide (20), another aliquot was dissolved in methanol, and  $^3\text{H}$  and  $^{14}\text{C}$  activity determined.

Radioactivity ( $^3\text{H}$  and  $^{14}\text{C}$ ) was determined by liquid scintillation counting (Mark III, Tracor Analytic Inc., Elk Village, MD); Quench correction was applied by the external standard method. The samples were counted for a sufficient length of time so that the error was  $\pm 3\%$ .

## RESULTS

**Plasma cholesterol concentration.** The average HDL and LDL esterified cholesterol concentrations of the subjects studied are shown in Table I. Throughout each experimental period the lipoprotein cholesterol

concentrations showed little variation as reflected by the small standard errors. Moreover, there were no detectable trends in lipoprotein cholesterol concentrations during the short course of these experiments.

**Injection of lipoproteins labeled with free cholesterol.** Subjects 1A, 2A, and 3 each received free [ $^3\text{H}$ ]cholesterol-HDL and free [ $^{14}\text{C}$ ]cholesterol-LDL. The HDL free cholesterol, HDL esterified cholesterol, and LDL esterified cholesterol specific activity curves from a representative subject (1A) are shown in Fig. 1. The specific activity of the HDL esterified [ $^3\text{H}$ ]cholesterol in all three subjects built up rapidly and reached a maximum within 100–250 min. By contrast, the HDL esterified [ $^{14}\text{C}$ ]cholesterol did not reach its peak specific activity until  $\sim 300\text{--}500$  min. At their respective peak specific activities, neither the HDL  $^3\text{H}$ -ester nor the  $^{14}\text{C}$ -ester attained the specific activity of the plasma HDL free cholesterol. The  $^3\text{H}$  and  $^{14}\text{C}$  specific activities of LDL esterified cholesterol built up more slowly than the HDL esters and intersected the latter after  $\sim 1$  d.

Subjects 1B and 2B also received HDL and LDL labeled with free cholesterol, but the labels were reversed in the lipoproteins ( $^{14}\text{C}$ -HDL and  $^3\text{H}$ -LDL). The specific activities of HDL free cholesterol, HDL ester, and LDL ester from subject 1B are shown in Fig. 2. The qualitative features of the free and esterified cholesterol specific activity curves were similar to those obtained in the previous experiments (Fig. 1) and in subject 2B. Furthermore, in all five dual-labeled free cholesterol experiments (subjects 1–3), the label initially associated with the injected HDL free cholesterol appeared more rapidly in HDL esterified cholesterol when compared with the label in the injected LDL free cholesterol. These qualitative similarities, coupled with the identical quantitative results obtained for  $^3\text{H}$  and  $^{14}\text{C}$  data in the kinetic analysis described below, exclude the occurrence of isotope effects or of other unknown effects due to biologic unreliability of free [ $^3\text{H}$ ]cholesterol (21).

**Injection of HDL or LDL labeled with esterified and free cholesterol.** Subject 4A with a bile fistula and subject 5A with an intact enterohepatic circuit both received dual-labeled HDL containing esterified [ $^3\text{H}$ ]cholesterol and free [ $^3\text{H}/^{14}\text{C}$ ]cholesterol. The specific activity curves are shown in Figs. 3 and 4. The features of the  $^{14}\text{C}$  specific activity curves were similar to the subjects shown in Figs. 1 and 2. The  $^{14}\text{C}$  activity in HDL esters increased rapidly and reached a peak within 200 min, at which time the  $^{14}\text{C}$  specific activity of HDL free cholesterol was much higher than the  $^{14}\text{C}$  HDL esters. By contrast, the HDL esterified [ $^3\text{H}$ ]cholesterol specific activity decayed during the entire time period and remained above that of the HDL free [ $^3\text{H}$ ]cholesterol from 30 min to the end of

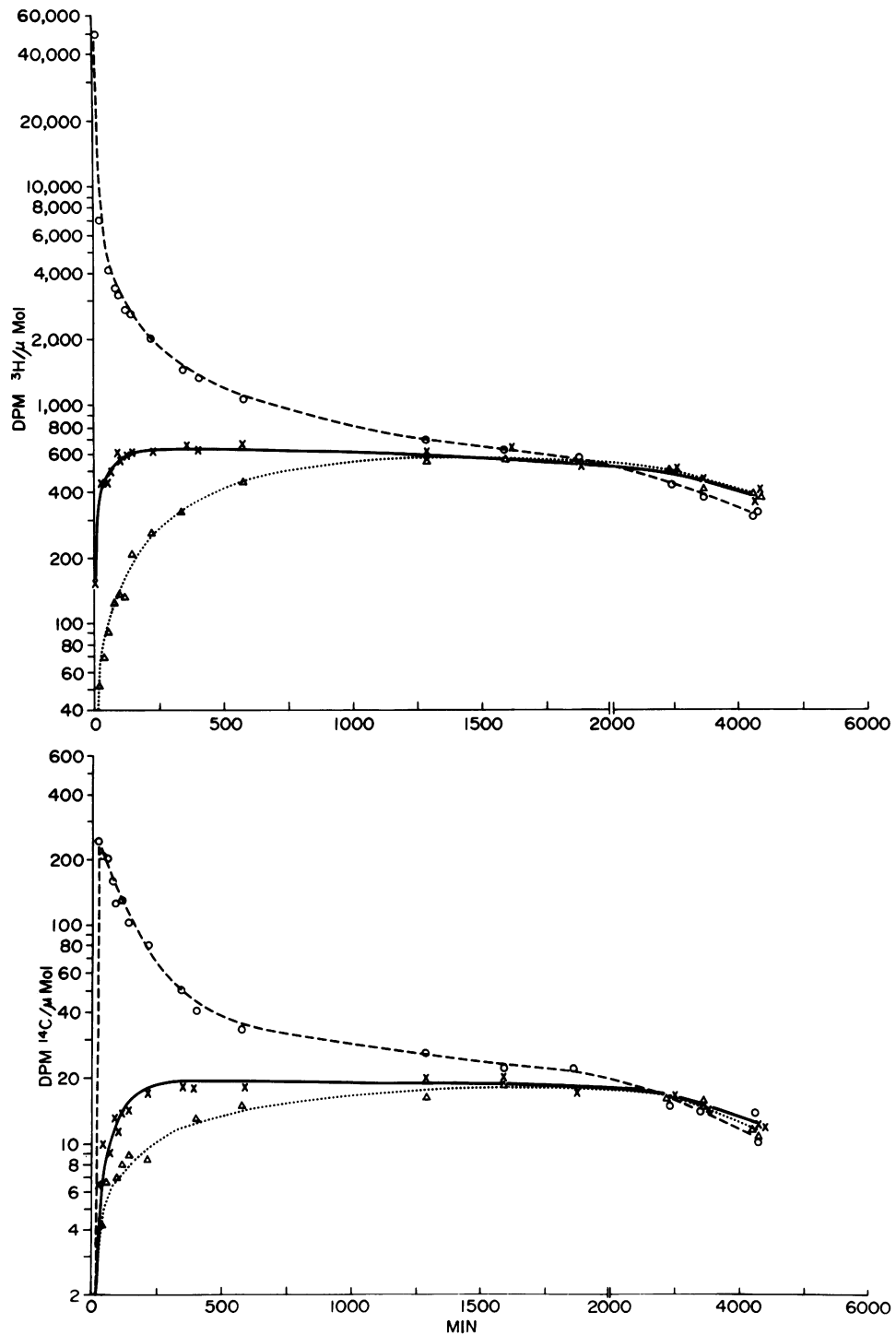


FIGURE 1 Patient 1A. Representative specific activity time course after simultaneous administration of HDL free [1,2-<sup>3</sup>H]cholesterol (above) and LDL free [4-<sup>14</sup>C]cholesterol (below). X, observed HDL esterified cholesterol values; O, observed plasma (HDL) free cholesterol values; Δ, observed LDL esterified cholesterol values. The solid lines represent the predictions for the HDL esterified cholesterol values derived from the simultaneous fitting of the <sup>3</sup>H and <sup>14</sup>C data to the model shown in Fig. 8. The dashed lines represent the forcing function for the plasma (HDL) free cholesterol. The dotted lines represent the forcing function for the LDL esterified cholesterol.

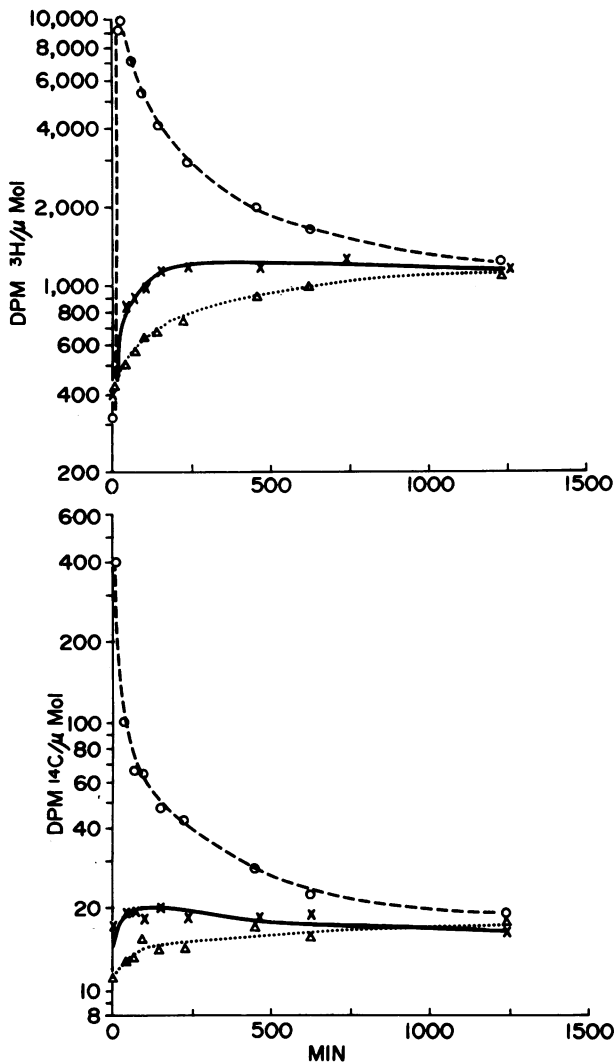


FIGURE 2 Patient 1B. Representative specific activity time course after simultaneous administration of HDL free [4- $^{14}\text{C}$ ]cholesterol (below) and LDL free [1,2- $^3\text{H}$ ]cholesterol (above). See Fig. 1 legend for description of symbols and lines.

the experimental periods. The specific activities of HDL esterified [ $^3\text{H}$ ]cholesterol in the blood samples obtained 10 min after injection of the  $^3\text{H}$ -labeled HDL preparations were within 5% of the theoretical initial specific activity calculated from the injected dose of label and the estimated HDL ester pool sizes. In both subjects 4A and 5A (Figs. 3 and 4), the  $^3\text{H}$ -LDL ester specific activities surpassed the  $^3\text{H}$ -free cholesterol specific activities within 12 h, whereas the  $^{14}\text{C}$ -LDL esters took  $>24$  h to surpass the  $^{14}\text{C}$ -free cholesterol specific activities. This observation suggests transfer of HDL ester to LDL, either directly or indirectly via VLDL.

It should be emphasized that the  $^3\text{H}$ -HDL ester specific activity curves in subjects 4A and 5A represent

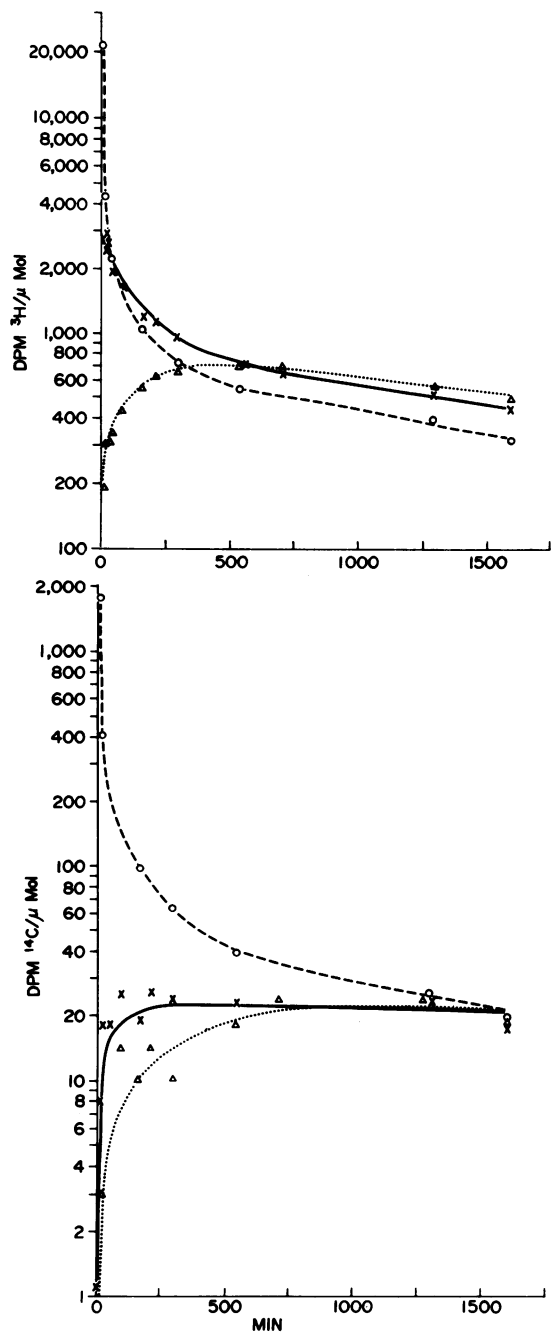


FIGURE 3 Patient 4A. Specific activity time course after administration of HDL labeled with esterified [1,2- $^3\text{H}$ ]cholesterol, free [4- $^{14}\text{C}$ ]cholesterol, and free [1,2- $^3\text{H}$ ]cholesterol.  $^3\text{H}$  data above;  $^{14}\text{C}$  data below. See Fig. 1 legend for description of symbols and lines.

a composite of decay from the administered  $^3\text{H}$ -HDL ester and input of new labeled ester from  $^3\text{H}$ -HDL free cholesterol as well as from other possible sources. The  $t_{1/2}$  of the initial  $^3\text{H}$ -HDL ester decay gives a rough approximation of HDL ester turnover. This value was

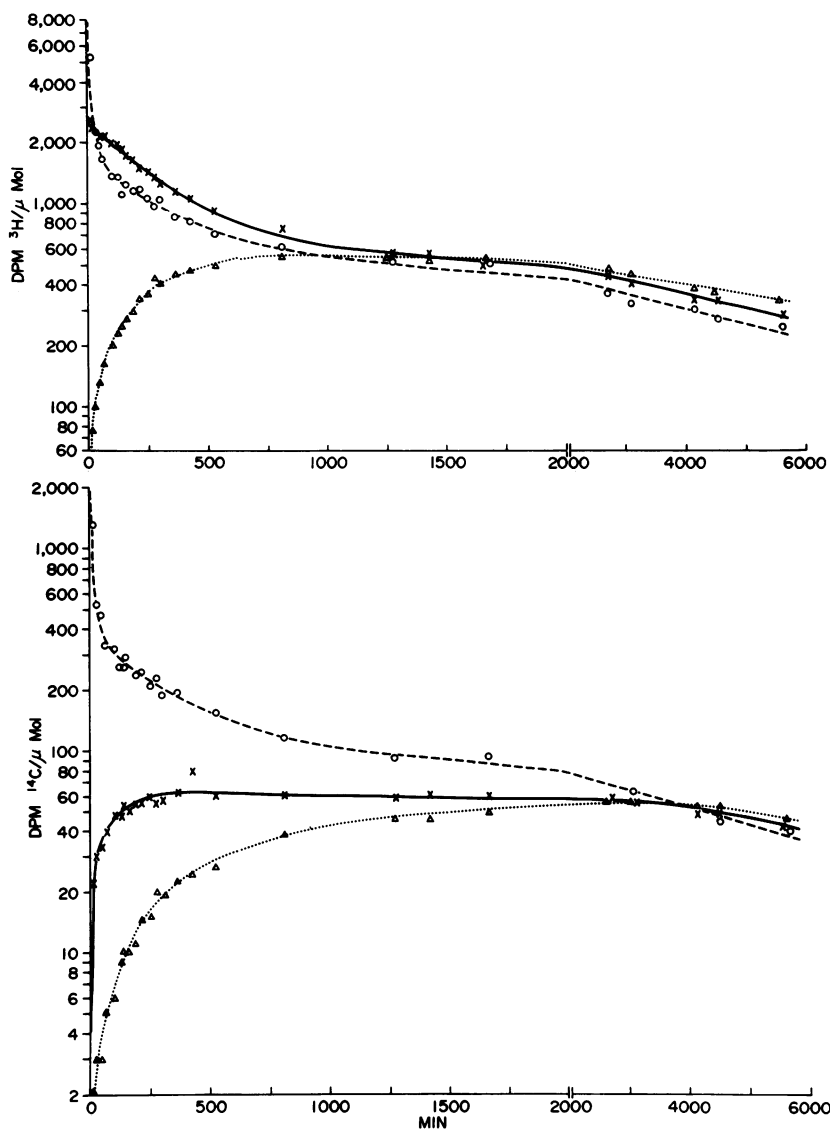


FIGURE 4 Patient 5A. Specific activity time course after administration of HDL labeled with esterified [1,2-<sup>3</sup>H]cholesterol, free [4-<sup>14</sup>C]cholesterol, and free [1,2-<sup>3</sup>H]cholesterol. <sup>3</sup>H data above; <sup>14</sup>C data below. See Fig. 1 legend for description of symbols and lines.

~2 h in subject 4A and 3.5 h in subject 5A. This rapid turnover of HDL esters could not be anticipated from the qualitative features observed after the administration of labeled free cholesterol or mevalonic acid.

Subject 4B was administered dual-labeled LDL containing esterified [<sup>3</sup>H]cholesterol and free [<sup>3</sup>H/<sup>14</sup>C]cholesterol. The specific activity curves are shown in Fig. 5. The <sup>14</sup>C specific activity time curves were similar to those obtained in the other subjects injected with LDL labeled free cholesterol. The specific activities of the LDL esterified [<sup>3</sup>H]cholesterol in blood samples collected at 7 and 12 min after the injection of the labels were virtually identical, and were within 5%

of the calculated initial specific activity. Although the experiment was of a short duration (320 min), certain qualitative features are readily discernible from the <sup>3</sup>H specific activity curves. Most notable were the rapid build-up of <sup>3</sup>H activity in the HDL ester fraction and rapid equilibration (at 3 h) of the LDL and HDL esterified [<sup>3</sup>H]cholesterol specific activities.

*Injection of [<sup>3</sup>H]mevalonic acid with or without particulate free [<sup>14</sup>C]cholesterol.* Subject 5B received [<sup>3</sup>H]mevalonic acid and had an intact enterohepatic circuit. Subject 6 received particulate [<sup>14</sup>C]cholesterol and [<sup>3</sup>H]mevalonic acid on two occasions, namely, when his enterohepatic circuit was open (6A) and in-



tact (6B). The specific activity data from subject 5B are shown in Fig. 6, and representative data for subject 6 are shown in Fig. 7. In all three experiments, build-up of radioactivity in the plasma free cholesterol after [ $^3\text{H}$ ]mevalonic acid administration was rapid and reached a peak in  $\sim 200$  min and then decayed in a multiexponential fashion. The build-up of radioactivity ( $^3\text{H}$  and  $^{14}\text{C}$ ) was more rapid in HDL ester than LDL ester. The shape of the plasma free [ $^{14}\text{C}$ ]cholesterol specific activity curves was variable, as has been noted previously, after the *in vivo* administration of labeled particulate cholesterol (11, 13).

The HDL and LDL esterified cholesterol were obtained from all subjects by ultracentrifugation of plasma at  $4^\circ\text{C}$  over 48 h, during which time *ex vivo* exchange of esters could have taken place. To explore this possibility blood samples were taken hourly during the initial 8 h in both subjects 3 and 5A and a portion of each sample treated immediately with heparin- $\text{MnCl}_2$  (15) to preclude *ex vivo* exchange, and another portion subjected to routine ultracentrifugation. The  $\alpha$  (HDL)-lipoproteins were separated from the  $\beta$ -lipoproteins (purity verified by electrophoresis on agarose gels) within 15 min of venipuncture. In both subjects the  $^3\text{H}$  and  $^{14}\text{C}$  specific activities of the HDL esters were virtually identical to those in the  $\alpha$  (HDL)-lipoprotein esters; the LDL ester specific activities were slightly below those in the  $\beta$  (LDL plus VLDL)-lipoproteins, as would be expected. The plasma concentrations of  $\alpha$ -esters and HDL esters were virtually identical, which further suggests that at  $4^\circ\text{C}$  *ex vivo* exchange of esters is minimal.

*Development of the model.* The presence of a steady state during the experiments was assumed in the kinetic analysis and was evidenced by the constancy of the plasma lipoprotein cholesterol levels (Table I) and biliary lipid secretion rates. During the initial 12 h after the administration of the labeled compounds, the cholesterol specific activities in the various fractions change rapidly relative to one another. Precaution was therefore taken to eliminate the unknown effects of eating and of chylomicron input into the system during this critical period by fasting the subjects before the experiment as well as throughout the first 12 h of the experiment (6 h for subject 6B).

Throughout the kinetic analysis of each subject's experimental data, both  $^3\text{H}$  and  $^{14}\text{C}$  data were tested simultaneously with identical model parameters. It was assumed that the same parameters must fit both data sets for the solution to be considered valid. In addition, it was reasoned that the correct final model was the simplest one that could fit all the data generated from each of the labeled preparations (mevalonic acid, free cholesterol, and esterified cholesterol). Background radioactivity in the lipoprotein cholesterol fractions from previous experiments was determined

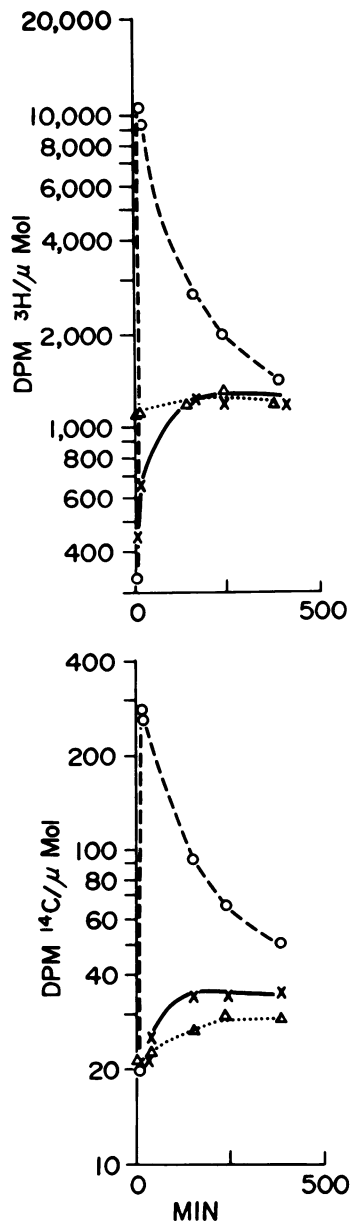


FIGURE 5 Patient 4B. Specific activity time course after administration of LDL labeled with esterified [ $1,2\text{-}^3\text{H}$ ]cholesterol, free [ $4\text{-}^{14}\text{C}$ ]cholesterol, and free [ $1,2\text{-}^3\text{H}$ ]cholesterol.  $^3\text{H}$  data above;  $^{14}\text{C}$  data below. See Fig. 1 legend for description of symbols and lines.

by obtaining two blood samples immediately before the administration of the next set of labeled compounds. The average specific activity in these background samples was used as the zero time specific activity in the kinetic analysis.

The HDL esterified cholesterol specific activity data could not be simulated with plasma (HDL or LDL) free cholesterol as its only precursor. Division of the

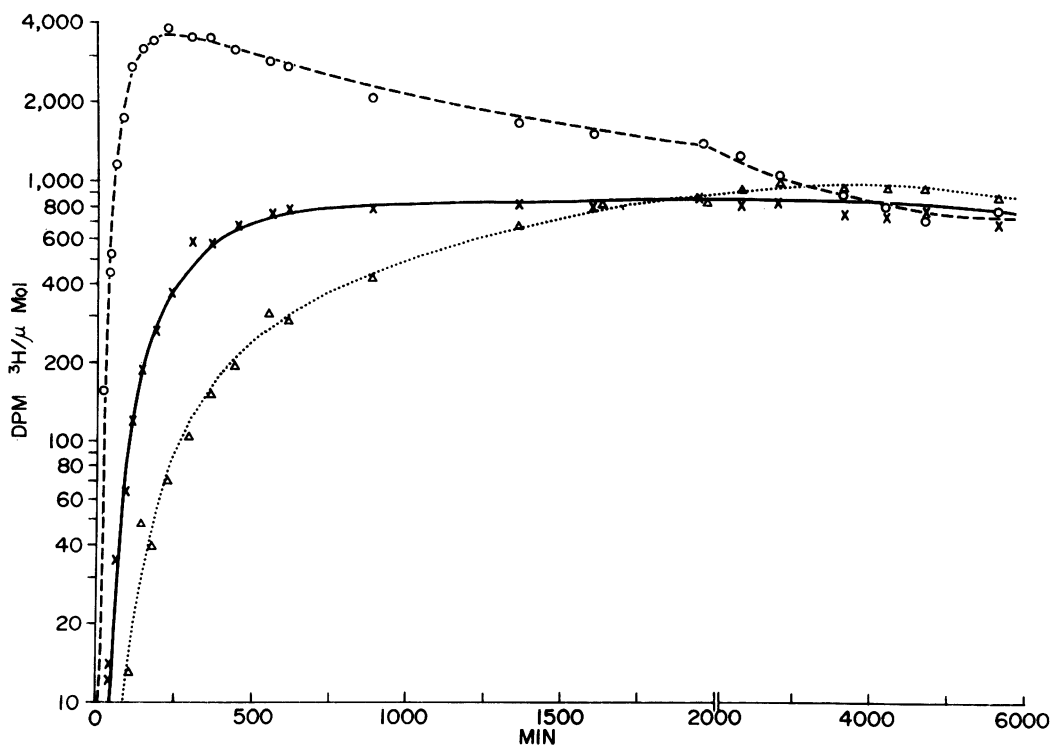


FIGURE 6 Patient 5B. Specific activity time course after administration of  $[5\text{-}^3\text{H}]$ mevalonic acid. See Fig. 1 legend for description of symbols and lines.

HDL esters into subcompartments as previously suggested (22) did not permit a fit of experiments 4A, 4B, or 5A. In these experiments, the administered lipoproteins were labeled with esterified  $[^3\text{H}]$ cholesterol and free  $[^3\text{H}/^{14}\text{C}]$ cholesterol. When the parameters were adjusted to allow a fit of the  $^{14}\text{C}$  data, the predicted  $^3\text{H}$  values were markedly deviant from the observed  $^3\text{H}$  values. Because of these discrepancies, subcompartments of HDL esterified cholesterol were not considered essential to fit the data and it was concluded that plasma free cholesterol was not the only immediate precursor of HDL esters.

The LDL ester fraction was therefore tested as an additional immediate precursor source for HDL esterified cholesterol (Fig. 8). The LDL ester source seemed reasonable since recent reports (6, 23) have indicated that there is *in vitro* exchange of cholesterol esters between HDL and LDL in man that is mediated by the cholesterol ester exchange protein. This approach was successful in simulating the HDL cholesterol ester data obtained in all 11 experiments without the need for HDL subcompartments. When the VLDL cholesterol ester specific activity data was substituted for LDL ester, the HDL ester specific activities could be successfully simulated in all experiments except subject 4B. However, the flux rates were inordinately high—the total mass of esterified cholesterol in the

HDL esterified cholesterol compartment [C(9)] lost from C(9) per minute was  $>50 \mu\text{mol}/\text{min}$ , and  $R(9, 4)$  was  $>10 \mu\text{mol}/\text{min}$ —and not physiologically compatible. Some transfer of VLDL ester to HDL is not excluded, but it was not found to be essential.

In subjects 1, 2, and 3, HDL free  $[^3\text{H}$  (or  $^{14}\text{C})]$ cholesterol and LDL free  $[^{14}\text{C}$  (or  $^3\text{H})]$ cholesterol were simultaneously administered. Differences between the initial rise of  $^3\text{H}$  and  $^{14}\text{C}$  activity in the HDL esters were apparent (Figs. 1 and 2), i.e., the label administered with HDL appeared faster in the esters. The cause of these differences could be resolved, and both the  $^3\text{H}$  and  $^{14}\text{C}$  ester data could be simulated during this initial phase by using the HDL free cholesterol along with LDL esters as precursors of the HDL esters, but not by using LDL free cholesterol or total plasma free cholesterol. This is compatible with the finding that when *ex vivo* free cholesterol exchange is prevented there are large differences between HDL and LDL free cholesterol specific activities which persist for 30–60 min after the administration of HDL (or LDL) labeled free cholesterol (24). Since HDL was isolated by ultracentrifugation in most subjects, *ex vivo* exchange was not always prevented in the present study. This problem could be circumvented when necessary, however, by extrapolating the HDL free cholesterol specific activity to zero at time zero when la-

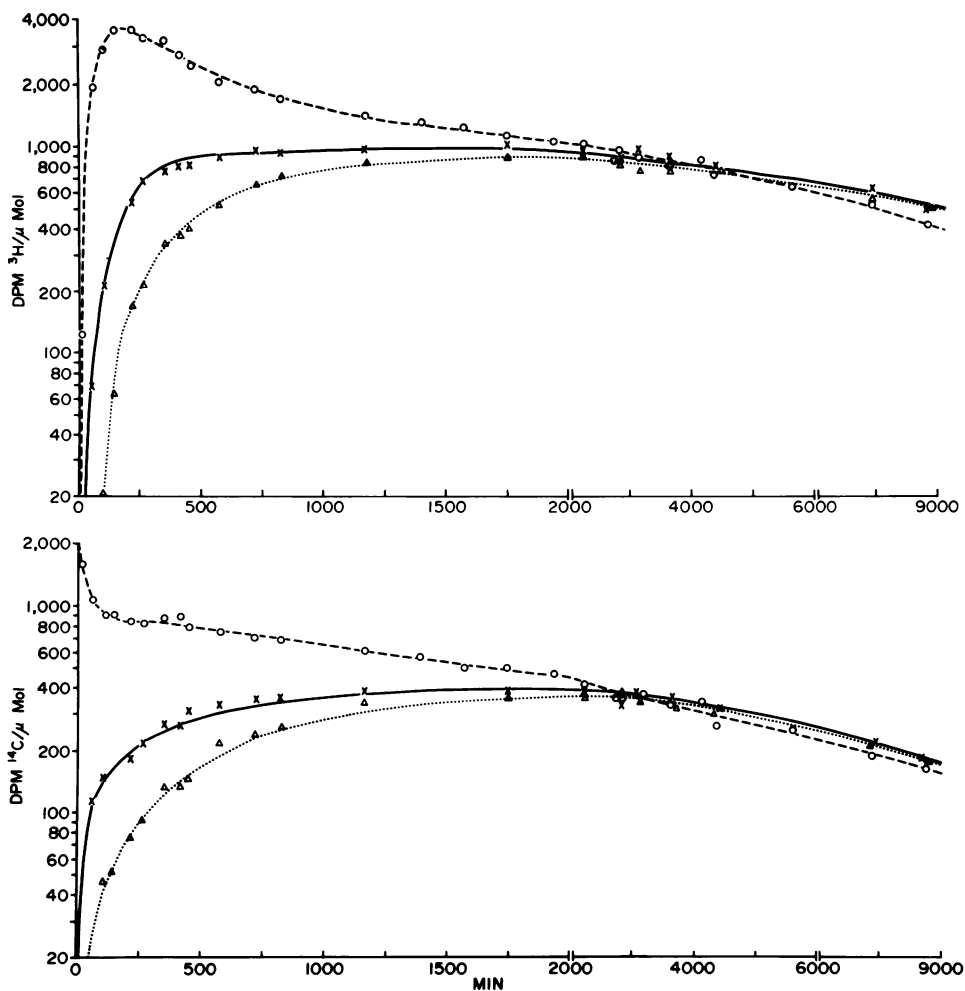


FIGURE 7 Patient 6A. Representative specific activity time course after simultaneous administration of [5-<sup>3</sup>H]mevalonic acid (above) and [4-<sup>14</sup>C]cholesterol (below). See Fig. 1 legend for description of symbols and lines.

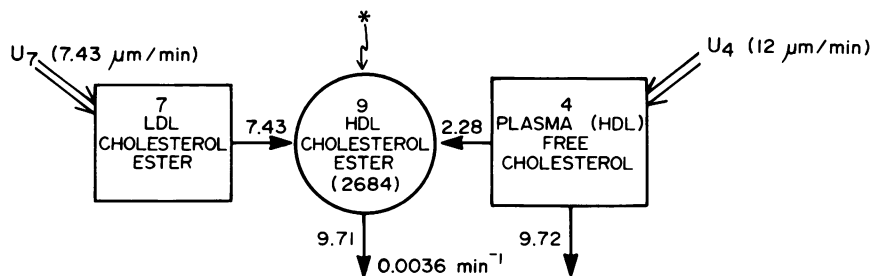


FIGURE 8 The HDL model, including parameter values from a representative patient (1A). \*, amount of radioactive HDL esterified cholesterol administered, when appropriate;  $U_4$ , rate of entry of cholesterol into compartment 4;  $U_7$ , rate of entry of cholesterol into compartment 7 necessary to fit C(9). The boxes around compartments 7 and 4 denote forcing functions in these compartments (which were not modeled). Fluxes (in micromoles per minute) are above the arrows. The value in parenthesis inside C(9) represents compartment size in micromoles.  $U_7$  varied to correspond to  $R(9, 7)$ .  $U_4$  was fixed arbitrarily to 12  $\mu\text{mol}/\text{min}$ , and the steady state was maintained via the pathway  $L(0, 4)$ .

beled LDL free cholesterol was administered, and to the calculated theoretic value, based on dose and HDL free cholesterol pool size, when labeled HDL was given (see dashed lines, Figs. 1–5). This approach was validated in subject 5A, in whom *ex vivo* free cholesterol exchange was prevented in several plasma samples, and in the other subjects by the finding of virtually identical results for HDL ester kinetics using the extrapolation method and using reconstructed HDL free cholesterol specific activity curves based on previously determined parameters (24).

**Parameters of the model.** The HDL cholesterol ester rate constants and steady state fluxes and pool sizes derived from compartmental analysis are shown in Table III. The overall rate constant of HDL ester, the fraction of esterified cholesterol in C(9) that leaves C(9) per minute, ranged from 0.0019 to 0.0083 min<sup>-1</sup> or on a daily basis the HDL ester pool turned over 2.7 to 12 times. The values obtained from the two experiments on each subject were in close agreement. It is also of interest that the values were similar on subject 6, who had both an open and closed enterohepatic circuit during the two experimental periods. HDL esterified cholesterol pool size, the steady-state mass of esterified cholesterol in C(9), could be determined from the kinetic information in subjects 4A and 5A, since <sup>3</sup>H-labeled HDL ester was administered in these experiments. The computer predicted pool sizes were in close agreement with the observed values in both subjects. The derived fluxes between the compartments expressed as micromoles per minute are also

shown in Table III. The rate of mass flow from HDL free cholesterol to HDL ester, R(9, 4), represents newly synthesized HDL cholesterol esters. There was little variation in R(9, 4) within each subject or from subject to subject, regardless of the administered labeled preparation or status of the enterohepatic circuit. The average flux rate from free cholesterol in micromoles per minute for the 11 experiments was 2.36±0.15 (mean±SE). This is equal to 3,384 μmol or 1,300 mg of HDL ester synthesized/d. The bulk of the mass flux into HDL was from LDL ester, R(9, 7), which ranged from 2 to 7 times that of the flux from free cholesterol. There was considerable variation between subjects in the magnitude of flux from the LDL source, but there was little variation within subjects. The partitioning of the HDL ester sources averaged 78% from LDL esters and 22% from free cholesterol. The total steady state flow into (and out of) the HDL ester compartment averaged 11.3 μmol/min or a net flux of 6.2 g/d.

## DISCUSSION

The results of this study show that in man the turnover of HDL esterified cholesterol occurs at a rate of ~6 times/d. In comparison HDL apoprotein A turnover rate is <0.4/d (25). Although physically located in the core of the HDL particle, the esters exist in a very dynamic state. This can be attributed to the presence of the plasma cholesterol ester transfer protein(s) and transfer of the HDL esters to VLDL and LDL (23, 26). In animal species that lack transfer protein activity the

TABLE III  
Rate Constants, Steady State Fluxes, and Pool Sizes Derived from Compartmental Analysis

Experiment No.	L(9, 9)	M(9)* observed	M(9)† calculated	R(9, 4)	R(9, 7)‡	R(9, 7)/R(9, 4)	R(9, 9)
	min <sup>-1</sup>	μmol	μmol	μmol/min	μmol/min		μmol/min
1A	0.0036±0.15 <sup>  </sup>	2,684.	—	2.28±0.05 <sup>  </sup>	7.43±0.19 <sup>  </sup>	3.25	9.71±0.15 <sup>  </sup>
1B	0.0042±0.21	2,684.	—	2.53±0.08	8.62±0.26	3.40	11.15±0.21
2A	0.0033±0.09	1,916.	—	2.12±0.04	4.27±0.12	2.01	6.39±0.09
2B	0.0032±0.07	1,916.	—	1.61±0.04	4.38±0.08	2.73	5.99±0.07
3	0.0019±0.15	3,295.	—	1.97±0.03	4.13±0.21	2.10	6.10±0.15
4A	0.0058±0.10	2,850.	3,042±0.06 <sup>  </sup>	2.75±0.11	14.86±0.09	5.41	17.61±0.07
4B	0.0071±0.56	2,850.	—	2.51±0.20	17.83±0.61	7.11	20.33±0.56
5A	0.0032±0.03	1,879.	1,826±0.03	1.69±0.04	4.11±0.05	2.43	5.80±0.04
5B	0.0042±0.10	2,290.	—	2.45±0.01	7.10±0.10	2.90	9.54±0.07
6A	0.0079±0.11	1,725.	—	2.80±0.08	10.66±0.08	3.81	13.46±0.07
6B	0.0083±0.16	2,145.	—	3.21±0.08	14.70±0.18	4.58	17.91±0.16

L(9, 9), fraction of esterified cholesterol in C(9) that leaves C(9) per minute; M(9), steady-state mass of esterified cholesterol in C(9), in micromoles.

\* Derived from plasma HDL ester concentration (Table I) and plasma volume as 4.5% of kg body weight. This observed value for M(9) was then used in the compartmental analysis to constrain the size of the HDL ester pool.

† Determined kinetically in experiments 4A and 5A only.

‡ Identical to U<sub>7</sub>.

<sup>||</sup> Parameter ± fractional standard deviation, i.e., SD divided by parameter value.

turnover of HDL cholesterol ester is slow, paralleling that of apoprotein HDL (27). The rapid turnover rate of HDL esters in man implies important metabolic functions in the regulation of LDL and VLDL metabolism. This property of HDL would allow for a readily available supply of esterified cholesterol needed by ester-poor nascent VLDL particles, which are secreted at fluctuating rates during the day. Rapid HDL ester turnover would also promote rapid equilibration of esterified cholesterol between all lipoprotein classes; this equilibration of esters is not seen in disorders in which HDL is absent or abnormal in structure, such as in Tangier's disease (28) or LCAT deficiency (29).

The kinetic analysis showed that in man HDL cholesterol esters are derived from two immediate sources, namely HDL free cholesterol and LDL esters. The partitioning is in favor of LDL esters as an immediate source by a molar ratio of  $\sim 3.5$  to 1 over free cholesterol. Direct transfer of LDL esters to HDL has been previously demonstrated in man in vitro (6, 23) and in rabbits in vivo (30), and is attributed to the presence of the cholesterol ester transfer protein found in the lipoprotein free portion of plasma (31). The observed rate of transfer of LDL esters to HDL in the present study was exceedingly rapid, averaging  $8.9 \mu\text{mol}/\text{min}$ . These LDL derived esters were probably originally synthesized in HDL via the LCAT reaction. The esters would then enter LDL indirectly through VLDL and IDL as intermediates, or directly from HDL (7, 23). Preliminary kinetic analysis of LDL esters in man suggests that the LDL-to-HDL flux is greater than the reverse flux from HDL directly to LDL.<sup>2</sup>

The other immediate source of HDL esters was HDL free cholesterol. This source contributed 22% of the total HDL ester input, and averaged  $2.4 \mu\text{mol}/\text{min}$  (range 1.6–3.2). This source represents the rate of plasma synthesis of HDL esters and is somewhat lower than the values reported for the in vitro rate of esterification by plasma LCAT. The in vitro rates have been reported (32, 33) to be between 50 and 100 nmol/liter/h; for a 70-kg man this would be  $\sim 2.6$ – $5.2 \mu\text{mol}/\text{min}$ . Since the synthesis of HDL esters in this report is indicative of the actual in vivo rate of HDL ester synthesis, a question can be raised as to whether LCAT rates are an accurate index of *de novo* HDL cholesterol ester formation in man. However, direct comparison of the two rates have not been made. This could have important implications with respect to disorders of HDL ester metabolism; these disorders may not be appreciated using the in vitro LCAT assay to determine HDL ester synthesis.

No insight was gained into the possible heteroge-

neity of the cholesterol esters in HDL, although heterogeneity has been amply demonstrated with respect to HDL apoprotein composition, lipid composition, and function. The kinetic analysis did not require the existence of subpools of HDL esters. The fit of the HDL ester data was excellent in the model shown in Fig. 8 and was not improved by the presence of HDL ester subcompartments. Although subcompartments of HDL esters may exist in vivo, either the design of these experiments does not allow for their distinction or very rapid exchange exists between the ester subcompartments masking their detection. Furthermore, the analysis could not exclude the possibility that the HDL esters are actually synthesized in a distinct LCAT/apoprotein A/transfer protein complex (34) rather than in HDL itself. The analysis clearly revealed, however, that the newly synthesized esters very rapidly appear in HDL and that the substrate for the reaction is HDL free cholesterol.

In this study, HDL cholesterol ester metabolism has been defined and quantitated. Five labeled precursors of HDL esters and labeled HDL ester itself were administered. Two labeled compounds were administered simultaneously in 10 of the 11 experiments to rigorously constrain the kinetic analysis since both labels ( $^3\text{H}$  and  $^{14}\text{C}$ ) were fit to identical parameters. This was especially useful in the subjects administered HDL (or LDL) esterified [ $^3\text{H}$ ]cholesterol and free [ $^3\text{H}/^{14}\text{C}$ ]cholesterol. By simultaneously modeling the  $^{14}\text{C}$  and  $^3\text{H}$  data, the  $^{14}\text{C}$  data effectively allowed for the separation of the injected free  $^3\text{H}$  from the esterified [ $^3\text{H}$ ]cholesterol. These latter experiments were crucial in that they permitted unique definition of the structure of the HDL esterified cholesterol model.

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