Comparison of exchange of α-tocopherol and free cholesterol between rat plasma lipoproteins and erythrocytes

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Abstract The simultaneous exchange of [^{3}H]tocopherol and [^{14}C]cholesterol between rat plasma, rat plasma lipoproteins, and RBC was studied in vitro to compare quantitatively (*a*) the fractional exchange rates and (*b*) the half-times for isotope equilibration. In all incubations of RBC with plasma or with plasma lipoprotein fractions, [^{14}C]cholesterol approached equilibrium more rapidly than [^{3}H]tocopherol. When the RBC contained the initial radioactivity, the half-times for equilibration with plasma of cholesterol and of tocopherol were 1.0 and 2.2 hr, respectively. However, the fractional exchange rates ($K_{RBC \rightarrow plasma}$) were 0.097/hr for cholesterol and 0.188/hr for tocopherol, indicating that the RBC tocopherol pool is turning over almost twice as rapidly as the RBC cholesterol pool.

The rat plasma lipoproteins were separated into five fractions by successive ultracentrifugation. Only two fractions, the high density lipoproteins (d 1.063–1.21) and the very low density lipoproteins (d < 1.006), participated to a significant extent in the exchange of either tocopherol or cholesterol with RBC. Cholesterol exchange between individual rat plasma lipoproteins and RBC had the same half-times for isotope equilibrium for the very low and high density lipoproteins, and the RBC fractional exchange rates were proportional to the amount of cholesterol in the lipoproteins. In tocopherol exchange between individual rat plasma lipoproteins and RBC, the very low density lipoprotein tocopherol did not equilibrate completely with the RBC. However, the initial rate of tocopherol exchange appeared to be the same for very low and high density lipoproteins. The very low density lipoproteins were disrupted by repeated freezing and thawing or by dehydrating and rehydrating, and analysis of the resulting lipoproteins indicated that free cholesterol was associated more closely than tocopherol with the phospholipid–protein portion of the molecule, which is thought to be on the surface. This difference in distribution of tocopherol and free cholesterol within very low density lipoproteins could account for their different rates of exchange and for the nonequilibrium of tocopherol between RBC and very low density lipoproteins.

The metabolism of cholesterol has been extensively studied in man (1, 2) and other species (3, 4). This ubiquitous sterol is present in the diet and can also be synthesized by almost all tissues of the body. Free cholesterol exchanges rapidly between RBC and plasma, with a half-time for radioactive equilibration ($t_{1/2}$) of about 1 hr (5), and also very quickly with other tissues in the body (3). Studies of plasma cholesterol specific activity in humans after the administration of intravenous radioactive cholesterol have indicated the presence of at least two pools of cholesterol in the body, one of which equilibrates more rapidly than the other (1, 2, 6–8). The more rapidly equilibrating pool is thought to include plasma, RBC, liver, and possibly intestine, with the slower equilibrating pool consisting of peripheral tissue such as muscle and skin (8). The physiological importance of the physical exchange of free cholesterol between plasma lipoproteins and body tissues in this scheme has not been evaluated, although Glomset (9) has postulated that physical exchange coupled with the action of lecithin:cholesterol acyltransferase (LCAT) upon the free cholesterol of the lipoproteins, particularly high density lipoproteins (HDL), could play a major role in the regulatory mechanism that determines cholesterol turnover and levels in tissues.

In contrast to cholesterol, α-tocopherol (the most biologically active component of vitamin E) is not synthesized in any animal tissues and must be ingested. The two molecules, however, are similar in many respects, including molecular weight, hydrogen, carbon, and oxygen content, and solubility properties. Both are present in RBC membranes and plasma lipoproteins, and previous studies on the exchange of tocopherol between rat erythrocyte membranes and whole plasma indicated that rapid exchange occurred, with a $t_{1/2}$ similar to that of cholesterol (10, 11). Orally

Abbreviations: VLDL, very low density lipoprotein(s); LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); TLC, thin-layer chromatography; LCAT, lecithin:cholesterol acyltransferase; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

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administered radioactive tocopherol also appears rapidly in plasma and tissues of rats and chickens (12).

HDL are the major fraction of total rat plasma lipoproteins; the very low density lipoproteins (VLDL) are the next largest fraction, and the low density lipoproteins (LDL) are present in quite small amounts (13). The precise location of free cholesterol and tocopherol in these lipoproteins is not known, although recent evidence has been presented supporting a model for VLDL that consists of a core of triglyceride and cholesteryl ester covered by a surface coat of protein, phospholipid, and free cholesterol (14, 15). It has been suggested that HDL and VLDL are assembled in the liver by similar mechanisms and that their basic structures are similar (16). Thus, the HDL may also have a surface coat of polar lipid with a nonpolar core. VLDL is involved in the transport of triglycerides from intestine and liver to plasma, where the subsequent action of lipoprotein lipase releases free fatty acid for tissue uptake. In contrast, HDL contains little triglyceride and serves as the major site of plasma cholesterol esterification by LCAT (17). The present study was undertaken to compare quantitatively the rates of exchange of cholesterol and tocopherol between the individual rat plasma lipoproteins and RBC. In this study, rat VLDL and HDL were compared directly to determine whether these lipoproteins had different activities in this exchange. The results of this study provide information on the individual role of VLDL and HDL in lipid metabolism and offer further suggestions regarding the location of cholesterol and tocopherol in the lipoprotein.

METHODS AND MATERIALS

D-α-[5-*methyl*-^{3}H]Tocopherol, obtained from Amersham/Searle, Arlington Heights, Ill., was purified by TLC on silica gel G plates, using benzene–absolute ethanol 99:1, prior to its use. Unlabeled α-tocopherol was added to the α-[^{3}H]tocopherol before TLC to adjust the specific activity to 5–10 $\times$ 10^6 dpm/μg. This stock solution of radioactive tocopherol in benzene was stored at −20°C and rechecked periodically by TLC to maintain purity > 95%. DL-α-[3,4-^{14}C]Tocopherol was generously supplied by Hoffmann-La Roche, Basle, Switzerland. DL-α-[3,4-^{14}C]-Tocopherol was prepared from its acetate by mild KOH hydrolysis in ethanol followed by addition of water and extraction with hexane. The final product (3×10^4 dpm/μg) was then purified as described above. [4-^{14}C]Cholesterol was obtained from Amersham/Searle and purified prior to use by TLC on silica gel G in petroleum ether–diethyl ether–acetic acid 90:10:1. DL-α-Tocopherol was generously supplied by Hoffmann-La Roche, Nutley, N.J. Precoated and prescored silica gel G plates were purchased from Analtech, Newark, Del., and were activated at 110°C for 1 hr prior to use. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer, model 3375, using Liquifluor (New England Nuclear Corp., Boston, Mass.) in toluene as the scintillation solvent and Bray's solution for aqueous samples.

Rat plasma lipoprotein fractions were prepared by the standard method of flotation ultracentrifugation (18) using KBr to adjust the density of the plasma. The plasma was centrifuged sequentially at densities of 1.006, 1.035, 1.063, and 1.21 according to the procedure used by Windmueller and Levy (13) to separate rat plasma lipoproteins. According to this scheme, the resulting five fractions contain the following lipoproteins. The first fraction (d < 1.006) contains very low density lipoproteins, the second (d 1.006–1.035) contains pure low density lipoproteins although not the total amount, the third (d 1.035–1.063) contains both low and high density lipoproteins, the fourth (d 1.063–1.21) contains the major amount of high density lipoproteins, and the fifth (d > 1.21) contains the plasma proteins and the very high density lipoproteins. Although it is agreed that rat low and high density lipoproteins do overlap to some extent in their densities, the exact densities reported for this separation vary somewhat in the literature (19, 20). The most recent study of this problem found that the majority of rat LDL lies in the density range 1.040–1.050 (20). The ultracentrifugations were carried out at 4°C in a Spinco model L at 100,000 *g* using a 40 rotor. The isolated lipoproteins were dialyzed against a phosphate buffer, pH 7.4, containing 0.01 M Na_2HPO_4, 0.124 M NaCl, and 0.3 mM Na_2EDTA.

Analyses

Cholesterol was extracted from RBC in chloroform–isopropanol as described by Rose and Oklander (21). Plasma and lipoprotein fractions (after dialysis) were extracted in chloroform–methanol 2:1 and purified by the procedure of Folch, Lees, and Sloane Stanley (22). Extracts of plasma lipoprotein fractions and erythrocytes were used for TLC, total cholesterol analysis, and radioactivity measurement. Total cholesterol of the plasma, lipoprotein fractions, and erythrocytes was assayed using the method of Franey and Amador (23). Prior to TLC, the chloroform aliquots of the plasma, lipoprotein fractions, and RBC extracts were taken to dryness under nitrogen and redissolved in a small volume of chloroform. The samples were then applied quantitatively to precoated silica gel G plates, which were then developed in petroleum ether–diethyl ether–acetic acid 90:10:1. After development, the plates were sprayed with an aqueous solution of rhodamine G, 0.002%, and viewed under ultraviolet light. Free and esterified cholesterol were identified from the R_f of standards; they were eluted from the silica gel with three washes of chloroform–methanol 4:1, and aliquots of the eluates were used for total cholesterol and radioactivity analyses. Remaining areas of the silica

gel plates containing other lipids were scraped and the silica gel was counted directly. Recovery of cholesterol was greater than 95%. Triglycerides were determined by the method of Van Handel and Zilversmit (24) and phospholipids by the method of Chen, Toribara, and Warner (25), using the purified chloroform extract mentioned above. Protein was assayed by the method of Lowry et al. (26).

Tocopherol from red cells and from plasma was extracted and assayed by the method of Kayden, Chow, and Bjornson (27). Aliquots of the plasma, lipoprotein fractions, and erythrocyte extracts were measured for radioactivity, and the specific activity of tocopherol was calculated.

All extracts were first dried under nitrogen and then counted in toluene scintillation solution. Samples of aqueous supernate were counted directly in Bray's solution (28). Quench corrections were made using an external standardization method. In double-label experiments, appropriate corrections were made for the spillover of ^{14}C into the ^{3}H channel.

In vitro and in vivo labeling of plasma and lipoproteins

Plasma LCAT was inactivated by either heating the plasma at 56°C for 30 min (29) or incubating it at 37°C with DTNB (5,5′-dithiobis-2-nitrobenzoic acid; Eastman Kodak, Rochester, N.Y.) at a concentration of 1 mg/ml for 2 hr followed by dialysis against buffer (30). Both procedures were shown to completely inactivate the enzyme so that subsequent overnight incubations at 37°C with these plasmas (both unlabeled, and labeled by the in vivo and in vitro methods) showed no conversion of cholesterol to cholesteryl ester, as determined by either mass or radioactivity measurement after TLC. However, heating the plasma frequently produced a cloudiness and a flocculent precipitate; for this reason the DTNB method was used throughout this study.

25-ml Erlenmeyer flasks were silanized by the addition of 4 ml of a 10% toluene solution of hexamethyldisilazane (Applied Science, State College, Pa.), stoppered, and left for at least 4 hr. The flasks were then rinsed three times (acetone, water, and acetone) and dried under nitrogen.

Radioactive tocopherol (1 μg, 10 $\times$ 10^6 dpm ^{3}H) and cholesterol (1 μg, 1 $\times$ 10^6 dpm ^{14}C) were added to the silanized flask in 0.5 ml of benzene and evaporated to a thin film along the bottom by rotating the flask under nitrogen at room temperature. 5 ml of rat plasma was then added to the flasks and incubated at 37°C for 4 hr in a shaking water bath. Incorporation of the two isotopes into plasma was similar and ranged from 20 to 40% of the initial amount of radioactivity.

The plasma was then transferred to an empty silanized flask and allowed to incubate an additional 8 hr in a shaking water bath at 37°C. This additional incubation period was necessary to permit complete equilibration of the radioactive cholesterol and tocopherol between the VLDL and HDL. This plasma was then used directly as the "in vitro"-labeled plasma or alternatively was incubated with washed erythrocytes for 3–4 hr to prepare "in vitro"-labeled erythrocytes. The "in vitro"-labeled individual lipoproteins were also prepared from this plasma by flotation ultracentrifugation. The lipoprotein lipid composition and electrophoretic mobility in agarose gel were unchanged by these incubations. The RBC also appeared normal when viewed under a light microscope, and hemolysis was minimal.

Treatment of animals

Female Sprague-Dawley rats weighing between 300 and 350 g were used. Blood was collected from the abdominal aorta after ether anesthesia, with EDTA as the anticoagulant. For in vivo labeling, radioactive tocopherol and cholesterol were dissolved in benzene, evaporated almost to dryness under nitrogen in ice, and dissolved in a mixture of olive oil and oleic acid 1:1; 0.2–0.3 ml of this solution, containing about 100 $\times$ 10^6 dpm [^{3}H]tocopherol (10 μg) and 5 $\times$ 10^6 dpm [^{14}C]cholesterol (10 μg), was fed to the rats by gastric tube, and the blood was collected after 16 hr.

A group of 12 rats was maintained on a high carbohydrate diet (ICN Nutritional Biochemicals Corp., Cleveland, Ohio) for 2 wk in order to increase the amount of VLDL present in plasma; triglyceride levels increased from about 50 to 250 mg/100 ml.

In vitro incubations of RBC with lipoproteins

All the incubations of RBC with plasma of the individual lipoprotein fractions were carried out in 50-ml Erlenmeyer flasks at 37°C in a water bath with gentle shaking. The RBC, either labeled or unlabeled, were washed three times with 15 vol of cold isotonic phosphate buffer (see above) before incubation with plasma or lipoprotein fractions. Each incubation mixture usually contained 5 ml of washed RBC (packed cells), 5 ml of plasma or lipoprotein fraction, and glucose (1 mg/ml of incubation mixture). 0.5-ml aliquots were taken at zero time, after 0.5 hr, and at hourly intervals up to 7–8 hr, with one or two aliquots taken later (12 or 24 hr). The aliquots were centrifuged, and the radioactivity in 100 μl of supernate was measured by liquid scintillation spectrometry. The RBC remaining were washed three times with isotonic phosphate buffer and extracted with isopropanol–chloroform, and the extract was evaporated before counting.

Disruption of VLDL

Samples of rat plasma VLDL were subjected to repeated freezing and thawing, dehydration–rehydration, and sonication procedures to disrupt the lipoprotein in an attempt to achieve a partial separation of polar and nonpolar components. After disruption, the samples were handled in a uniform manner. The samples were immersed in a dry ice–

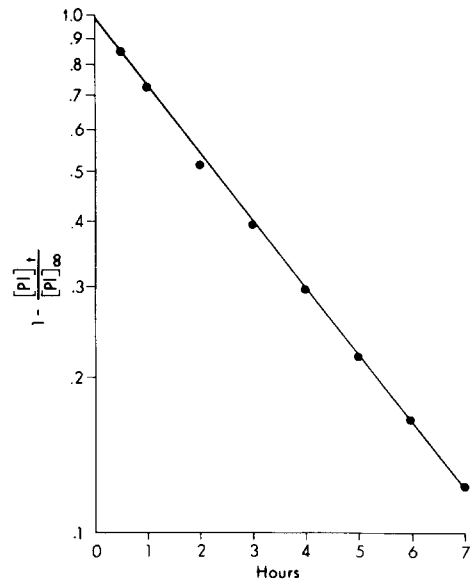

Fig. 1. Semilogarithmic plot of $(1 - [Pl]_t/[Pl]_\infty)$, where $[Pl]_t$ is the amount of [^{14}C]cholesterol (dpm/ml) in the plasma at time t and $[Pl]_\infty$ is the radioactivity at equilibrium (infinite time).

acetone bath for 10 min and then immersed in a water bath at 37°C for 20 min. This freeze–thaw cycle was repeated 10 times in the course of a day, and the resulting material was then centrifuged at 100,000 *g* overnight. In the dehydration–rehydration treatment, the sample was evaporated to dryness on a rotary evaporator while immersed in a 37°C water bath. After drying, distilled water was added to make up the original volume. This cycle was carried out five times and the sample was then centrifuged overnight at 100,000 *g*. These procedures are essentially the same as used by Zilversmit (31) to prepare coat and core material from chylomicrons. The third method used to disrupt the VLDL was sonication of the sample for periods of 2 min followed by cooling in an ice bath for 5 min for a total of 10 min sonication time at tap 4 (Sonifier, model W140; Heat Systems-Ultrasonics, Plainview, N.Y.). Centrifugation overnight at 100,000 *g* was then carried out.

After each of the above treatments, the VLDL solutions became visibly more opaque. The final centrifugation step separated material on the basis of density: unchanged VLDL floated to the top of the tube along with particles having a protein/lipid ratio less than or equal to VLDL and material with a protein/lipid ratio greater than the original VLDL migrated to the bottom of the tube.

TABLE 1. Distribution of tocopherol in rat plasma lipoproteins[a]

	A[b] % Tocopherol Mass	B[c] % [^{3}H]Tocopherol (in vivo)	C[d] % [^{3}H]Tocopherol (in vitro)
VLDL (d < 1.006)	21	23	23
LDL (d 1.006–1.035)	3	2	2
LDL+HDL (d 1.035–1.063)	5	6	5
HDL (d 1.063–1.21)	64	61	64
Proteins + VHDL (d > 1.21)	7	8	6

[a] Values for lipoproteins are averages of duplicate assays, which agreed within 10% for VLDL and HDL and within 30% for the remaining fractions. For this group of 18 rats the RBC tocopherol level was 4.1 ± 0.3 μg/ml of packed cells (average ± SEM) and plasma was 9.7 ± 0.5 μg/ml.

[b] Percentage distribution of tocopherol mass in untreated plasma.

[c] Percentage distribution of [^{3}H]tocopherol after feeding tocopherol (in vivo technique). The specific activity of tocopherol in the in vivo-labeled whole blood was 5200 ± 200 dpm/μg for plasma and 5100 ± 300 dpm/μg for the RBC. Tocopherol concentrations in VLDL and HDL were 2.3 and 5.9 μg/ml of plasma, respectively.

[d] Percentage distribution of [^{3}H]tocopherol after incubation of the plasma with tocopherol dispersed on silanized glass (in vitro technique). The specific activity of tocopherol in the in vitro-labeled plasma was 45,100 ± 600 dpm/μg. Tocopherol concentrations in VLDL and HDL were 2.2 and 6.1 μg/ml of plasma, respectively.

Calculations

In both single- and double-label experiments, appropriate quench curves were used to calculate the disintegrations per minute (dpm) of the samples. Rates of exchange of tocopherol and cholesterol between red cells and plasma or plasma lipoprotein fractions were calculated using a modification of the formulas of Wallach, Riezenstein, and Bellavia (32) for exchange between two compartments in a closed system.

In all exchange studies, time sequence concentrations in dpm/ml of supernates and erythrocytes are designated as $[Pl]_t$ (t_1, t_2, t_3, etc.) or $[RBC]_t$, and equilibrium concentrations as $[Pl]_\infty$ and $[RBC]_\infty$. The experimental values of $(1\text{-}[Pl]_t/[Pl]_\infty)$ or $(1\text{-}[RBC]_t/[RBC]_\infty)$, when plotted semilogarithmically against time, gave a regression curve from which the slope and half-time of the exchange could be determined. An example of the calculations involved and a plotted regression curve are shown in **Fig. 1.** The slope, in turn, is used to calculate the flux coefficient from *red blood cell* to *plasma* or *lipoprotein fraction* by Eq. 1:

$$K_{RBC \rightarrow Pl} = \frac{(\text{slope})[Pl]_\infty(1 - \text{hematocrit})/(\text{hematocrit})}{[RBC]_0} \quad \text{Eq. 1}$$

where $[Pl]_\infty$ is the dpm/ml of plasma or fraction at infinite time and $[RBC]_0$ is the dpm/ml of packed red blood cells at zero time. In Eq. 1 the RBC are labeled and the plasma or lipoprotein fraction is unlabeled at zero time. In this case the slope is obtained by plotting ln $(1\text{-}[Pl]_t/[Pl]_\infty)$ vs. time. The flux coefficient from plasma to RBC ($K_{Pl \rightarrow RBC}$) can be calculated from this data by Eq. 2:

$$K_{Pl \rightarrow RBC} = (\text{slope} - K_{RBC \rightarrow Pl}) \frac{(1 - \text{hematocrit})}{\text{hematocrit}} \quad \text{Eq. 2}$$

When the situation is reversed and the plasma or lipoprotein fraction contains the radioactivity and the RBC are unlabeled at zero time, Eq. 3 is used to calculate the flux coefficient from plasma to RBC:

$$K_{Pl \rightarrow RBC} = \frac{(\text{slope})[RBC]_\infty(\text{hematocrit})/(1 - \text{hematocrit})}{[Pl]_0} \quad \text{Eq. 3}$$

where $[RBC]_\infty$ is the dpm/ml of packed red blood cells at infinite time and $[Pl]_0$ is the dpm/ml of plasma or fraction at zero time. It should be noted that this flux constant is equivalent to the fractional exchange rate of the pool, i.e., $K_{RBC \rightarrow Pl}$ equals that fraction of the pool in the RBC that exchanges with plasma per unit time. $K_{RBC \rightarrow Pl}$ can be calculated from this data by Eq. 4:

$$K_{RBC \rightarrow Pl} = (\text{slope} - K_{Pl \rightarrow RBC}) \frac{\text{hematocrit}}{(1 - \text{hematocrit})} \quad \text{Eq. 4}$$

RESULTS

Incorporation of [^{3}H]tocopherol and [^{14}C]cholesterol into rat plasma lipoproteins

Table 1 shows the distribution of [^{3}H]tocopherol in rat plasma lipoproteins in plasmas that were labeled initially with [^{3}H]tocopherol by the in vivo and the in vitro techniques; the mass distribution in untreated plasma is also shown. The unlabeled plasma and the in vivo- and in vitro-labeled plasmas were separated simultaneously into their respective lipoprotein fractions by sequential ultracentrifugation at the densities indicated. Tables 1 and 2 present the results of an experiment with a group of 18 rats. The pooled plasma of 12 of these animals was divided into two portions; one was used to prepare cold lipoprotein fractions and the other portion was incubated with [^{3}H]tocopherol and [^{14}C]cholesterol that had been dispersed in a silanized flask. Lipoprotein fractions were prepared from both plasma pools. The remaining six rats were fed [^{3}H]tocopherol and [^{14}C]cholesterol by gastric tube, blood was collected 16 hr later, and the isolated plasma was pooled and separated into lipoprotein fractions, i.e., "in vivo-labeled lipoproteins." It was found, in separate determinations, that the specific activity of tocopherol in the HDL was the same as in the VLDL for both the in vivo and in vitro techniques, indicating that tocopherol was equilibrated between the individual lipoproteins. Data for the other three lipoprotein fractions indicated that they were also in equilibrium with very low and high density lipoproteins, although measurement of tocopherol mass in the fraction was difficult because of the very small amounts present. The percentage distribution of tocopherol in the lipoproteins is essentially the same in all three plasmas (Table 1, columns A, B, and C). Therefore, (*1*) the in vivo and in vitro techniques for incorporation of [^{3}H]tocopherol into rat plasma lipoproteins can be used interchangeably, and (*2*) neither technique appears to alter the normal pattern of tocopherol distribution in untreated rat plasma.

Table 2 presents the same data for free cholesterol in rat plasma lipoproteins. In comparing columns A, B, and C, it is evident that the percentage distribution of free cholesterol measured quantitatively in untreated plasma (A) is the same as the percentage distribution of free cholesterol radioactivity when either the in vivo (B) or the in vitro technique (C) was used for incorporation of radioactivity. For cholesterol, it was possible to obtain accurate specific activities for all the fractions, and equilibration was observed between the lipoproteins for both the in vivo and in vitro methods of incorporation. In comparing Tables 1 and 2, it is apparent that the distribution of tocopherol and free cholesterol is similar in the lipoproteins; about 60% of each compound is present in the HDL (d 1.063–1.21) and about 20% is found in the VLDL (d < 1.006). Since a small amount of HDL is present at a density lower than 1.063, the actual amount of these lipids in HDL is somewhat higher. When rat plasma was incubated for 3–4 hr with α-[^{3}H]tocopherol and [^{14}C]cholesterol dispersed on silanized glass, about 20–40% of the ^{3}H and ^{14}C radioactivity was recovered in the plasma. This represents a considerable increase in radioactivity incorporation over that obtained in vivo, when only 1–3% of the total [^{3}H]tocopherol

TABLE 2. Distribution of cholesterol in rat plasma lipoproteins[a]

	A[b] % Free Cholesterol Mass	B[c] % Free [^{14}C]-Cholesterol (in vivo)	C[d] % Free [^{14}C]-Cholesterol (in vitro)
VLDL (d < 1.006)	19	18	20
LDL (d 1.006–1.035)	5	3	3
LDL+HDL (d 1.035–1.063)	11	13	14
HDL (d 1.063–1.21)	59	62	60
Proteins + VHDL (d > 1.21)	5	4	3

[a] Values for the lipoproteins are averages of duplicate assays, which agreed within 10%. For this group of 18 rats the total cholesterol level in RBC was 1700 ± 110 μg/ml of packed cells and in plasma was 710 ± 30 μg/ml (average ± SEM). The plasma free cholesterol concentration was 160 ± 11 μg/ml.

[b] Percentage distribution of free cholesterol mass in untreated plasma.

[c] Percentage distribution of [^{14}C]cholesterol after feeding (in vivo technique). The specific activity of free cholesterol in whole blood labeled in vivo was 75 ± 3 dpm/μg for plasma and 73 ± 3 dpm/μg for RBC. Free cholesterol concentrations for VLDL and HDL were 29 and 98 μg/ml plasma, respectively.

[d] Percentage distribution of [^{14}C]cholesterol after incubation of the plasma with cholesterol dispersed on silanized glass (in vitro technique). The specific activity of free cholesterol of the in vitro-labeled plasma was 220 ± 12 dpm/μg. Free cholesterol concentrations of VLDL and HDL were 31 and 95 μg/ml of plasma, respectively.

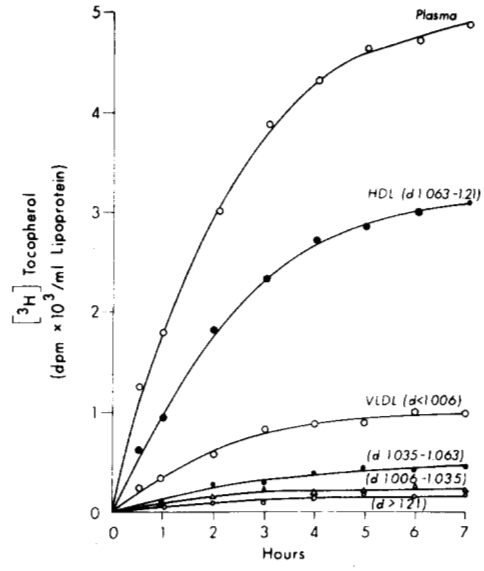

Fig. 2. Incorporation of radioactivity into rat plasma and rat plasma lipoproteins during incubations with [^{3}H]tocopherol-labeled RBC. Hematocrits were 38% for all incubations and the temperature was 37°C.

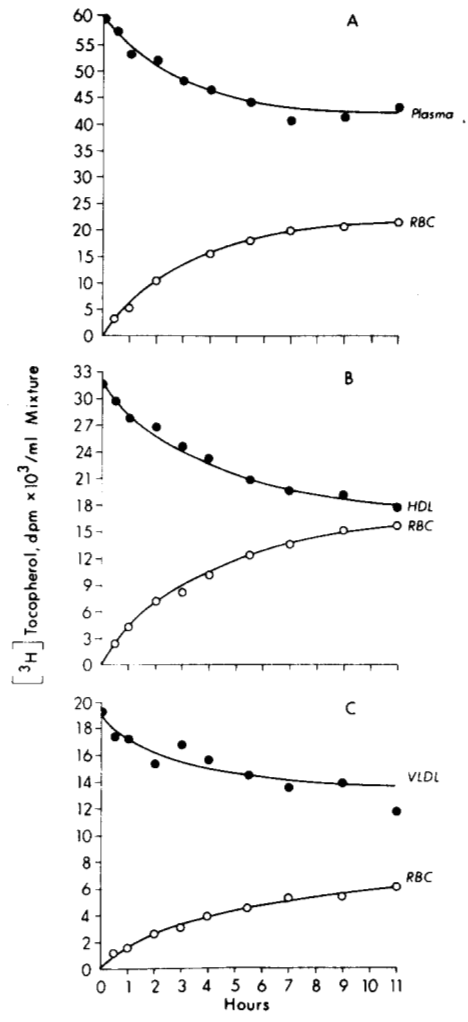

Fig. 3. Exchange of [^{3}H]tocopherol between rat plasma or rat plasma lipoproteins and RBC, with the radioactivity initially in (*A*) plasma, (*B*) HDL, and (*C*) VLDL. Hematocrits were 39%, 39%, and 37% for the plasma, HDL, and VLDL incubations, respectively, and the temperature was 37°C.

and 5–10% of [^{14}C]cholesterol as free cholesterol are recovered in rat plasma after feeding the radioactive compounds to the animals. Analysis of rat RBC showed an average concentration of 3.9 μg of tocopherol and 1800 μg of cholesterol/ml of packed RBC, with a range of 3–7 and 1600–1850 μg/ml, respectively. Plasma values averaged 9.8 μg of tocopherol and 600 μg of total cholesterol/ml of plasma, with a range of 7–15 and 500–750 μg/ml, respectively. Plasma free cholesterol averaged 23% of the total cholesterol. In the plasma lipoprotein fractions, free cholesterol averaged 55% for VLDL, 44% for d 1.006–1.035, 30% for d 1.035–1.063, and 20% for HDL.

[^{3}H]Tocopherol and [^{14}C]cholesterol exchange between rat plasma lipoproteins and erythrocytes

The exchange of [^{3}H]tocopherol and [^{14}C]cholesterol between rat plasma lipoproteins and RBC was studied in experiments in which labeled materials were incorporated simultaneously into the lipoproteins or into the RBC by both the in vivo and in vitro techniques. The results were essentially the same for the fractional exchange rates, the $t_{1/2}$, and the slopes by both methods of incorporation. Whole rat plasma was first incubated with DTNB to inactivate the LCAT so that cholesterol esterification and mass transfer would not complicate the exchange studies. However, DTNB-treated and untreated plasma and lipoproteins were found to follow the same initial kinetics as determined from the slope of the regression plot.

[^{3}H]Tocopherol exchange. **Fig. 2** shows the results of tocopherol exchange when [^{3}H]tocopherol-labeled RBC were incubated with unlabeled rat plasma and lipoprotein fractions. In this case, each incubation mixture contained 4 ml of washed RBC and 4 ml of plasma or lipoprotein fraction so that the resulting hematocrit was 30–40%, or approximately the hematocrit of normal rat blood (40%). It is seen from Fig. 2 that the HDL (d 1.063–1.21), which contains about 60% of the total plasma tocopherol, exchanges about 60% as much [^{3}H]tocopherol as whole plasma with the same preparation of radioactive RBC. This would indicate that the tocopherol exchange between HDL and RBC is the same whether the HDL is isolated or is studied in the presence of the other plasma lipoproteins. For the determination of $[LP]_\infty$, at least one data point was taken in the 11–14-hr time period, and the curve from the shorter time periods was extended to include this point. It was observed that there was little change in [LP] after 12 hr, so this value, $[LP]_{12\,hr}$, was used as an estimate of $[LP]_\infty$.

Fig. 3 shows the results of a similar incubation except that [^{3}H]tocopherol was distributed in the plasma lipoproteins. Here, also, it is evident that the extent of exchange of

TABLE 3. Calculated kinetic parameters for the exchange of tocopherol between rat plasma lipoproteins and RBC after in vivo labeling, with [^{3}H]tocopherol present initially in (A) the RBC and (B) the plasma lipoproteins[a]

	Hematocrit	$t_{1/2}$[b]	Slope	$K_{LP\rightarrow RBC}$[c]	$F_{LP\rightarrow RBC}$[d]	Tocopherol: Lipoprotein	Tocopherol: RBC	$K_{RBC\rightarrow LP}$[e]	$F_{RBC\rightarrow LP}$
		hr	hr^{-1}	hr^{-1}	*μg/ml mix/hr*	*μg/ml mix*		hr^{-1}	*μg/ml mix/hr*
A. RBC ([^{3}H]tocopherol) ⇌ Plasma Lipoproteins									
Plasma	41	2.2	0.32	0.190	0.475	2.5	2.7	0.188	0.508
HDL (d 1.063–1.21)	41	2.4	0.29	0.242	0.387	1.6	2.7	0.122	0.329
VLDL (d < 1.006)	40	2.4	0.29	0.402	0.201	0.5	2.6	0.022	0.057
B. Plasma Lipoproteins ([^{3}H]tocopherol) ⇌ RBC									
				f				g	
Plasma	39	2.2	0.31	0.113	0.339	3.0	2.6	0.126	0.372
HDL (d 1.063–1.21)	39	3.3	0.21	0.113	0.192	1.7	2.6	0.062	0.161
VLDL (d < 1.006)	37	3.4	0.20	0.066	0.059	0.9	2.4	0.079	0.190

[a] Sections A and B present data from single incubations in two completely separate experiments. In A, pooled plasma from 10 rats was separated into individual lipoproteins and incubated with pooled, labeled RBC obtained from six rats fed the radioactive lipids by gavage. Plasma (labeled in vivo) was obtained similarly from another group of rats, fractionated, and incubated with pooled, unlabeled RBC from a group of 10 rats (section B).
[b] Calculated from the relationship $t_{1/2} = 0.693/\text{slope}$.
[c] Calculated from Eq. 2.
[d] Flux (F) = $K \times$ concentration of tocopherol in μg/ml of mixture.
[e] Calculated from Eq. 1.
[f] Calculated from Eq. 3.
[g] Calculated from Eq. 4.

radioactivity from labeled HDL with RBC is greater than the labeled VLDL fraction. The primary data from this type of graph are not easily interpreted because each individual lipoprotein fraction has a different amount of total radioactivity initially. The actual kinetics of the exchange, i.e., how rapidly isotope equilibrium is approached between the RBC and the various plasma lipoproteins, are not obtainable directly from the curves in Figs. 2 and 3. However, using the standard system (32) it is possible to obtain the fractional exchange rate or flux constant, K, by plotting (semilogarithmically) the function $(1 - [PI]_t / [PI]_\infty)$ vs. time and calculating the slope of the resulting straight line.

These kinetic parameters are presented in Table 3. The slopes were calculated between the 1- and 3-hr periods and were observed to have less variation than the fractional exchange rate when hematocrit and pool sizes were changed. The slopes for the exchange of tocopherol were essentially the same for plasma and for VLDL and HDL, indicating that the rate of isotope equilibration is the same for these components between 1 and 3 hr. The fraction of tocopherol in RBC that is exchanged per hour with plasma ($K_{RBC\rightarrow PI}$), high density lipoproteins ($K_{RBC\rightarrow HDL}$), and very low density lipoproteins ($K_{RBC\rightarrow VLDL}$) is 0.188, 0.122, and 0.022, respectively. The $K_{RBC\rightarrow HDL}$ is about 60% of the value for $K_{RBC\rightarrow PI}$, which would be expected because HDL contains about 60% of the total plasma tocopherol. That $K_{RBC\rightarrow HDL}$ is 60% of $K_{RBC\rightarrow PI}$ is further evidence that isolated HDL exchanges tocopherol with the RBC at the same rate as HDL in whole plasma. The fractional exchange rate of tocopherol in RBC with VLDL ($K_{RBC\rightarrow VLDL}$), however, is only about 12% of that observed with plasma ($K_{RBC\rightarrow PI}$), whereas the concentration of VLDL tocopherol is about 20%. Measurement of tocopherol indicated that no net mass transfer occurred between RBC and VLDL; however, it appeared that equilibration of the [^{3}H]tocopherol between RBC and VLDL had not taken place. This point is illustrated in Table 4,

TABLE 4. Specific activity of [^{3}H]tocopherol after a 10-hr incubation of rat plasma lipoproteins and RBC, with initial radioactivity incorporated in vivo

Incubation Mixture	VLDL[a]	HDL[b]	Plasma	RBC
		dpm/μg		
VLDL(^{3}H) + HDL	3560	3120		
VLDL + HDL (^{3}H)	8200	8120		
VLDL (^{3}H) + RBC	5270			1470
VLDL + RBC (^{3}H)	2910			6790
HDL (^{3}H) + RBC		6570		5040
HDL + RBC (^{3}H)		5680		5120
Plasma (^{3}H) + RBC			7120	4960
Plasma + RBC (^{3}H)			4660	4620

[a] d < 1.006.
[b] d 1.063–1.21.

which gives the specific activities of [^{3}H]tocopherol determined after a 10-hr incubation of various combinations of lipoproteins with RBC from in vivo studies. Although tocopherol has essentially equilibrated or is approaching equilibrium in the incubations of VLDL with HDL, of HDL with RBC, and of plasma with RBC, it has clearly not reached equilibrium in the incubation of VLDL with RBC.

In Table 3, section A, the fractional exchange rates during incubations with RBC of tocopherol in plasma, HDL, and VLDL ($K_{LP \rightarrow RBC}$) were calculated from the appropriate slopes and $K_{RBC \rightarrow LP}$ by Eq. 2. The calculated flux of tocopherol from RBC to lipoproteins ($F_{RBC \rightarrow LP}$) and from lipoproteins to RBC ($F_{LP \rightarrow RBC}$) was similar for incubations of RBC and plasma, and RBC and HDL, but not for RBC and VLDL. These data indicate that the closed two-compartment model of analysis is not applicable to tocopherol exchange between RBC and VLDL. The data from Table 3, section A, represent an experiment in which the RBC were labeled with radioactive tocopherol in vivo. In six additional experiments (RBC labeled in vitro, three, and in vivo, three), similar data were obtained. In these experiments, the values for the slopes ranged from 0.270 to 0.420, from 0.195 to 0.385, and from 0.198 to 0.378 hr^{-1} for tocopherol exchange between RBC and plasma, RBC and HDL, and RBC and VLDL, respectively. The $K_{RBC \rightarrow LP}$ values ranged from 0.110 to 0.235, from 0.050 to 0.145, and from 0.018 to 0.040 for plasma, HDL and VLDL, respectively. The variations in fractional exchange rates observed between the separate experiments may arise from the variation in concentrations of both the individual RBC and lipoprotein molecules per milliliter of incubation mixture, and tocopherol per RBC and lipoprotein molecule, i.e., the number of tocopherol molecules per individual RBC and lipoprotein.

The slopes and fractional exchange rates for studies in which [^{3}H]tocopherol was initially in the plasma lipoproteins are shown in Table 3, section B. The slopes from the semilogarithmic plot of $(1 - [RBC]_t/[RBC]_\infty)$ vs. time for the VLDL and HDL are the same, indicating that the apparent approach to equilibrium, at least initially in the first 3 hr, is the same for the tocopherol that exchanges between these lipoproteins and the RBC. However, the $K_{VLDL \rightarrow RBC}$ (0.066 hr^{-1}) was lower than the $K_{HDL \rightarrow RBC}$ (0.113 hr^{-1}) because of incomplete equilibration of tocopherol between VLDL and RBC. The slope for the whole plasma was somewhat higher than that for the individual lipoproteins, but the fractional exchange rate was the same for plasma and for HDL ($K_{Pl \rightarrow RBC} = K_{HDL \rightarrow RBC} =$ 0.113 hr^{-1}). In the experiments in which the radioactivity was initially located in the plasma lipoproteins (Table 3, section B), the results are less accurate. One reason for this is that equilibration is not readily reached (Table 4), thus making estimations of concentrations at infinite time less precise. These experiments are also inherently less precise because, before counting, each RBC aliquot must be washed three times, then extracted, and the extract must be evaporated. The data presented in Table 3, section B, were calculated from an experiment in which plasma lipoproteins were labeled in vivo. The data are consistent with an experiment in which the plasma lipoproteins were labeled in vitro. In general, tocopherol flux calculations are consistent with a two-compartment, closed, steady state model in the incubations of RBC with plasma and of RBC with HDL, but not of RBC with VLDL. We attempted to analyze tocopherol exchange between RBC and VLDL with other mathematical models, such as plotting ln[VLDL] or ln(specific activity of tocopherol in VLDL) vs. time. This type of plot can be analyzed by curve peeling or computer simulation to yield information as to the number of pools, pool sizes, and turnover times (7). In these experiments, such approaches were not applicable because most of the data were collected in the initial 8 hr and equilibrium was not approached. It was observed that hemolysis usually started after 8 hr, and it is at this time that a slower-equilibrating pool would presumably be affecting the curve.

In comparing the range of kinetic parameters observed in the two different experiments, qualitative agreement is noted for the fractional exchange rates and slopes, except in the case of VLDL. Although these values are not quantitatively the same, the values in the second series of experiments, in which the label was initially in the lipoprotein, are not outside the range observed in the first group of experiments, in which the label was initially in the RBC.

[^{14}C]Cholesterol exchange. The exchange of free cholesterol between rat plasma lipoproteins and RBC was studied simultaneously with tocopherol exchange because the lipoproteins or the RBC contained both [^{14}C]cholesterol and [^{3}H]tocopherol. **Fig. 4** shows the exchange of [^{14}C]cholesterol between labeled rat RBC and unlabeled plasma lipoproteins. The pattern is similar to that observed for tocopherol (Fig. 2), with the HDL containing about three times as much [^{14}C]cholesterol as VLDL and the remaining fractions containing only small amounts of the radioactivity at equilibrium (6–8 hr). The sum of the [^{14}C]cholesterol in the lipoprotein fractions was found to approximate that of plasma at equilibrium, indicating that separating the individual lipoproteins has little effect on their exchange of cholesterol with RBC. **Fig. 5** shows the same experiments in which [^{14}C]cholesterol-labeled plasma lipoproteins are incubated with unlabeled, washed RBC. It is apparent that the [^{14}C]cholesterol equilibrates more rapidly than the [^{3}H]tocopherol (Fig. 3) with the RBC.

The calculated kinetic parameters for the exchange of cholesterol in these systems are shown in **Table 5.** In section A ([^{14}C]cholesterol initially in the RBC), the slopes from the semilogarithmic plot of $(1 - [LP]_t/[LP]_\infty)$ vs. time for VLDL and HDL are the same (0.6 hr^{-1}), indicat-

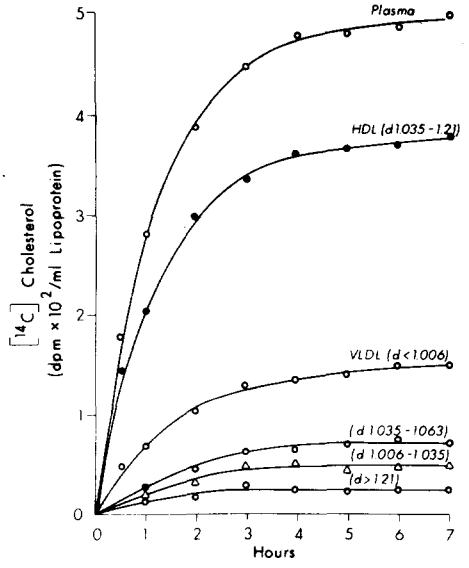

Fig. 4. Incorporation of radioactivity into rat plasma and rat plasma lipoproteins during incubations with [^{14}C]cholesterol-labeled RBC. Hematocrits were 38% for all incubations and the temperature was 37°C.

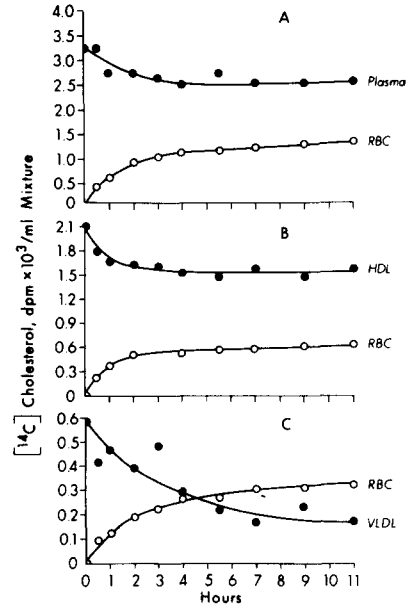

Fig. 5. Exchange of [^{14}C]cholesterol between rat plasma or rat plasma lipoproteins and RBC, with the radioactivity initially in (*A*) plasma, (*B*) HDL, and (*C*) VLDL. Hematocrits were 39%, 39%, and 37% for the plasma, HDL, and VLDL incubations, respectively, and the temperature was 37°C.

ing identical approach to equilibrium for [^{14}C]cholesterol exchanging between these lipoproteins and RBC. In addition, the $K_{RBC \rightarrow HDL}$ (0.063 hr^{-1}) is about 60% and the $K_{RBC \rightarrow VLDL}$ (0.019 hr^{-1}) about 20% of the $K_{RBC \rightarrow Pl}$ (0.097 hr^{-1}). Both of these results indicate that the fraction of RBC cholesterol exchanging with the lipoprotein per unit time is directly proportional to the amount of cholesterol present in the lipoprotein, because the VLDL and HDL contain about 20% and 60% of the plasma free cholesterol, respectively.

TABLE 5. Calculated kinetic parameters for exchange of cholesterol between rat plasma lipoproteins and RBC after in vivo labeling, with [^{14}C]cholesterol present initially in (A) the RBC and (B) the plasma lipoproteins[a]

	Hematocrit	*b* $t_{1/2}$	Slope	*c* $K_{LP \rightarrow RBC}$	*d* $F_{LP \rightarrow RBC}$	Free Cholesterol: Lipoprotein	Free Cholesterol: RBC	*e* $K_{RBC \rightarrow LP}$	*d* $F_{RBC \rightarrow LP}$
		hr	hr^{-1}	hr^{-1}	*μg/ml mix/hr*	*μg/ml mix*		hr^{-1}	*μg/ml mix/hr*
A. RBC ([^{14}C]cholesterol) ⇌ Plasma Lipoproteins									
Plasma	41	1.0	0.70	0.868	65.1	75	695	0.097	67.4
HDL (d 1.063–1.21)	41	1.2	0.60	0.773	41.0	53	695	0.063	43.8
VLDL (d < 1.006)	40	1.1	0.61	0.887	14.2	16	682	0.019	13.0
B. Plasma Lipoproteins ([^{14}C]cholesterol) ⇌ RBC				*f*				*g*	
Plasma	39	1.7	0.40	0.532	46.3	87	672	0.084	56.4
HDL (d 1.063–1.21)	39	1.7	0.41	0.366	20.9	57	672	0.028	18.8
VLDL (d < 1.006)	37	1.8	0.38	0.340	10.2	30	629	0.023	14.5

[a] Sections A and B contain data from single incubations in separate experiments. Refer to footnote *a* in Table 3 for details. Tables 3, A, and 5, A, present data from one experiment in which the RBC contained both [^{3}H]tocopherol and [^{14}C]cholesterol. Tables 3, B, and 5, B, present data from a separate experiment in which the plasma lipoproteins contained both radioactive lipids.

[b] Calculated from the relationship $t_{1/2} = 0.693/\text{slope}$.

[c] Calculated from Eq. 2.

[d] Flux (F) = $K \times$ concentration of free cholesterol in μg/ml of mixture.

[e] Calculated from Eq. 1.

[f] Calculated from Eq. 3.

[g] Calculated from Eq. 4.

TABLE 6. Analysis of tocopherol and cholesterol mass in RBC and plasma lipoproteins during in vitro incubation

Incubation Time	Plasma[a]	RBC[b]	HDL[a]	RBC[b]	VLDL[a]	RBC[b]
hr						
			Tocopherol[c]			
0	4.9	6.5	3.2	6.5	0.4	6.5
3	4.7	6.8	3.1	6.5	0.4	6.6
6	4.2	6.5	3.2	6.9	0.4	6.8
			Total Cholesterol[d]			
0	540	1800	420	1810	70	1800
3	550	1770	470	1760	90	1770
6	570	1680	460	1780	80	1820

[a] μg/ml of supernate.
[b] μg/ml of packed cells.
[c] Values for tocopherol are for single assays from an incubation of RBC and plasma or lipoproteins prepared from pooled blood of 12 rats. Hematocrits were 40% for all incubations. Experimental error in the assay for supernate is about 6% and for RBC is about 10%.
[d] Values for total cholesterol are for single assays from an incubation of RBC and plasma or lipoproteins prepared from pooled blood of 10 rats. Hematocrits were about 42% for all incubations. Experimental error in the assay for supernate is about 5% and for RBC is about 8%.

Table 5, section A, presents data from one experiment in which the RBC were labeled with [^{14}C]cholesterol. Similar data obtained in six additional experiments (the RBC were labeled in vivo in three and in vitro in three) appear to fit the closed, two-compartment, steady state model, i.e., the calculated flux in approximates flux out. The range observed for the slope values was from 0.510 to 0.910, from 0.390 to 0.721, and from 0.370 to 0.730 hr^{-1} for cholesterol exchange between RBC and plasma, RBC and HDL, and RBC and VLDL, respectively. The range observed in RBC fractional exchange rates ($K_{RBC \rightarrow Pl}$) was from 0.070 to 0.120, from 0.025 to 0.080, and from 0.015 to 0.040 hr^{-1} for plasma, HDL, and VLDL, respectively, and in lipoprotein ($K_{LP \rightarrow RBC}$) was from 0.490 to 0.980, from 0.350 to 0.920, and from 0.330 to 0.910 hr^{-1} for plasma, HDL, and VLDL, respectively.

Table 5, section B, presents the same data from experiments in which [^{14}C]cholesterol-labeled rat plasma lipoproteins were incubated with unlabeled, washed RBC. The values for the slopes for HDL (0.41 hr^{-1}) and VLDL (0.38 hr^{-1}) are similar, and the fractional exchange rates, $K_{HDL \rightarrow RBC}$ (0.37 hr^{-1}) and $K_{VLDL \rightarrow RBC}$ (0.30 hr^{-1}), are also similar, indicating that the VLDL and HDL free cholesterol pools are turning over at the same rate, even though approximately twice as much free cholesterol is exchanged between HDL and RBC as between the VLDL and RBC. The data in Table 5, section B, are from an experiment in which the plasma lipoproteins were labeled in vivo. Similar data were obtained from an experiment in which the plasma lipoproteins were labeled with [^{14}C]cholesterol in vitro. In comparing the two types of experiments (Table 5, sections A and B), it is seen that, despite deviations in fractional exchange rates of as much as 125%, the two-compartment model for flux calculations appears appropriate, i.e., flux in approximates flux out for each incubation. Quarfordt and Hilderman (34) observed deviations of up to 135% for lipoprotein fractional exchange rates in their studies of cholesterol exchange between human RBC and LDL and between RBC and HDL. In our experiments, duplicate incubations carried out at the same time with the same preparation of RBC and lipoproteins gave average deviations of fractional exchange rates of about 10%. It is thought that the larger deviations of this kinetic parameter between separate experiments is the result of changes in both the concentrations of the RBC and plasma lipoproteins and the concentrations of tocopherol and cholesterol in each RBC or lipoprotein.

The closed, two-compartment mathematical model that we have used requires that equilibrium be approached in the time period of observation and that no net mass transfer occur. That no net mass transfer was observed in these incubations within the experimental error of the assays is seen in two separate experiments shown in **Table 6.** In nine additional experiments, tocopherol and cholesterol were measured in both supernatant fractions and RBC at the beginning and at the end of a 10- or 12-hr incubation period. Although differences of the order of 10% were sometimes found in one pool or the other, there was no consistent change in the mass analyses of either pool. These results are in agreement with other studies on tocopherol (33) and free cholesterol (34) exchange between normal human RBC and individual plasma lipoproteins in which no net mass transfer was found. The specific activity of tocopherol in Table 4 indicates that equilibrium is approached in 10 hr in all incubations except that of RBC and VLDL. Since cholesterol approaches equilibrium more rapidly than tocopherol, it was found that after 6 hr cholesterol exchange is essentially complete. Specific activities at this time for a representative experiment in which RBC are labeled initially are: RBC, 14.2 ± 1.3 dpm/μg, and plasma, 12.6 ± 1.1 dpm/μg; RBC 16.5 ± 1.3 dpm/μg, and HDL, 17.1 ± 1.0 dpm/μg; and RBC, 17.5 ± 1.2 dpm/μg, and VLDL, 14.9 ± 1.5 dpm/μg.

Because free cholesterol is completely equilibrated between VLDL and RBC and tocopherol does not reach equilibrium even after an overnight incubation, it would appear that these lipids differ in either their location or their binding within the VLDL complex. The following experiments were carried out to compare tocopherol and cholesterol distribution in VLDL.

Rat plasma VLDL was subjected to various treatments, including freezing and thawing, dehydrating and rehydrating, and sonicating, to break up the molecule; all treat-

TABLE 7. Composition of rat plasma VLDL after disruptive treatment and ultracentrifugation (d 1.006) for 16 hr

Treatment of VLDL	Centrifugation[a]	Protein[b]		Phospholipid		Triglyceride		Total Cholesterol		Free Cholesterol		Esterified Cholesterol		Total Lipid[c]		Tocopherol: % [^{3}H]-Tocopherol	Tocopherol: Total	
		μg	%	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%		μg	%
1. Freeze and thaw	d < 1.006	336	48.0	313	58.0	1,903	93.0	529	78.0					2,930	84.0	84.0		
	d > 1.006	364	52.0	227	42.0	143	7.0	149	22.0					571	16.0	15.0		
2. Dehydrate and rehydrate	d < 1.006	203	29.0	108	20.0	1,882	92.0	380	56.0					2,503	73.0	78.0		
	d > 1.006	497	71.0	432	80.0	164	8.0	298	44.0					998	27.0	22.0		
3. Sonicate	d < 1.006	266	38.0	232	43.0	1,760	86.0	488	72.0					2,651	76.0	80.0		
	d > 1.006	434	62.0	308	57.0	286	14.0	190	28.0					851	24.0	20.0		
4. Freeze and thaw[d]	d < 1.006	574	51.3	490	43.3	4,051	90.9	980	71.9	485	60.8	495	87.6	5,864	79.8		14.1	78.4
	d > 1.006	545	48.7	641	56.7	406	9.1	383	28.1	313	39.2	70	12.4	1,480	20.2		3.9	21.6
5. Dehydrate and rehydrate[d]	d < 1.006	196	18.0	93	7.4	3,529	82.5	397	49.5	195	33.8	202	89.8	4,158	64.0		7.84	65.2
	d > 1.006	891	82.0	1,170	92.6	750	17.5	405	50.5	382	66.2	23	10.2	2,341	36.0		4.19	34.8
6 Freeze and thaw[e]	d < 1.006	1,965	57.7	775	36.1	15,425	94.7	1,090	74.0	680	66.9	410	88.6	17,577	87.0	88.0		
	d > 1.006	1,475	42.9	1,370	63.9	863	5.3	389	26.0	336	33.1	53	11.4	2,659	13.0	12.0		

[a] Centrifugation was carried out overnight at 100,000 *g* for 16 hr.

[b] Lipoproteins of d < 1.006 have a lipid/protein ratio greater than original VLDL, and lipoproteins of d > 1.006 have a lipid/protein ratio less than original VLDL.

[c] Total lipid was estimated by adding the amounts of phospholipid, triglyceride, and cholesterol, the cholesteryl ester portion being multiplied by 1.7 to account for its fatty acid side chain.

[d] Prior to treatment these samples were dialyzed against distilled water.

[e] A group of 12 rats was made hyperlipemic by maintaining them on a high carbohydrate diet for 2 wk.

ments were followed by centrifugation at 100,000 *g* for 16 hr. The freeze–thaw and dehydration–rehydration techniques have been used previously by Zilversmit and others (31, 35–37) to remove the polar phospholipid–protein coat from the more nonpolar triglyceride–cholesteryl ester core of chylomicrons, and the sonication technique has been used to disrupt plasma lipoproteins (38). The object of the experiment was to first separate the polar phospholipid–protein portion from the remainder of the VLDL and then to determine what the relative distributions of tocopherol and free cholesterol were in each portion. In experiments 1–3 of **Table 7** it is clear that some separation of polar and nonpolar VLDL constituents is taking place because there is a larger percentage of protein and phospholipid and a lower percentage of triglyceride and total cholesterol in the d > 1.006 fraction. This fraction is thought to consist of the more polar constituents located on the outside of the VLDL that are dislodged by freezing and thawing, etc. The d < 1.006 fraction would contain the partially stripped VLDL and any unchanged VLDL. The distribution of [^{3}H]tocopherol in experiments 1–3 (Table 7) is more nearly like that of the total lipid and quite different from that of the phospholipid and protein. This same observation is made in experiments 4 and 5, in which tocopherol mass was measured. In these two experiments it appears that the distribution of free cholesterol is in between that of phospholipid and tocopherol. In experiment 5, in which almost all of the phospholipid and protein is removed from the VLDL, it is observed that approximately two-thirds of the free cholesterol and only one-third of the tocopherol is removed with it. Thus, as a rough estimate it would appear that about two-thirds of the free cholesterol and about one-third of the tocopherol is located on the surface of the VLDL molecule in association with the phospholipid and protein components.

One group of rats was maintained on a high carbohydrate diet to increase their VLDL content in the plasma; plasma triglycerides increased from about 50 mg to 250 mg/100 ml. Data from the VLDL of these rats are shown in experiment 6 of Table 7, and it is seen that, here also, a larger percentage of the total VLDL free cholesterol is isolated with the protein–phospholipid portion (d > 1.006) than tocopherol. Although there is considerable variation in the amount of protein and phospholipid removed from rat plasma VLDL by these procedures, this difference between the distribution of free cholesterol and tocopherol has been consistently observed. Similar results have been obtained with rat lymph chylomicrons and VLDL mixtures.[2]

DISCUSSION

Earlier work on the in vitro exchange of tocopherol between plasma and RBC in rats has indicated that the ex-

[2] Bjornson, L. K., H. J. Kayden, and H. E. Gallo-Torres. Unpublished observations.

change is rapid and that the $t_{1/2}$ for isotope equilibration is close to that of cholesterol (10, 11). In the present study, in which tocopherol and cholesterol exchanges were examined simultaneously, it was found that although $t_{1/2}$ values for the two molecules were indeed similar, that for [^{14}C]cholesterol was always lower (1.0–1.8 hr) than that for [^{3}H]tocopherol (2.2–3.4 hr), indicating that cholesterol approaches equilibrium more rapidly than tocopherol. This finding was observed over a wide range of hematocrits and pool sizes for every incubation carried out with the various combinations of RBC and plasma or lipoproteins. This may reflect fundamental differences between these molecules in their interactions with the lipoproteins or in their location in the lipoprotein. Even though the cholesterol is equilibrating more rapidly than tocopherol between RBC and plasma, the RBC tocopherol pool ($K_{RBC \rightarrow Pl} = 0.188\ hr^{-1}$) is turning over almost twice as rapidly as the RBC cholesterol pool ($K_{RBC \rightarrow Pl} = 0.097\ hr^{-1}$). This is probably due, at least in part, to the large differences in pool size, for the RBC cholesterol pool is 400–500 times the size of the tocopherol pool. On the other hand, the free cholesterol in the plasma pool is both equilibrating and turning over more rapidly ($K_{Pl \rightarrow RBC} = 0.5\ hr^{-1}$) than the plasma tocopherol ($K_{Pl \rightarrow RBC} = 0.11\ hr^{-1}$), and the ratio of the concentration of free cholesterol to tocopherol in plasma is 13 to 16.

The analysis of the data for cholesterol exchange shows that our values for equilibration of half of the [^{14}C]cholesterol ($t_{1/2}$) between rat plasma and RBC are similar to the previously reported value of 1 hr (5). Recently, d'Hollander and Chevallier (39, 40) have attempted to quantitate the movement of cholesterol between rat plasma and RBC by following the exchange of free cholesterol and its esterification during incubation of whole blood and various combinations of plasma and washed RBC. They found that the cholesterol flux between plasma and RBC was not constant, ranging from 0.065 to 0.020 mg/hr/g of whole blood, and that the turnover time for erythrocyte cholesterol was 9.2 hr, calculated from the initial flux. This value is close to the turnover time of RBC cholesterol observed in our in vitro incubations of RBC and plasma, which was 10 hr (Table 5, section A). However, in view of the variation in fractional exchange rates from one experiment to the other, only a qualitative comparison of the in vitro turnover times with those of whole blood is warranted. The object of the present work was to compare the kinetics of exchange of tocopherol and cholesterol between rat RBC and plasma lipoproteins with the concentrations of these lipids in the RBC or lipoproteins within physiological limits.

In cholesterol exchange between rat plasma lipoproteins and RBC, the $t_{1/2}$ for VLDL and HDL and the fraction exchange rates ($K_{HDL \rightarrow RBC}$ and $K_{VLDL \rightarrow RBC}$) are the same. These results indicate that free cholesterol is associated with or bound to VLDL and HDL in the same manner. This similarity in binding is consistent with the proposal that the VLDL and HDL are assembled in the liver by a similar mechanism and that their structures are the same with respect to the distribution of the lipids; i.e., polar lipids, cholesterol and phospholipids, are on the surface and the nonpolar lipids, triglyceride and cholesteryl ester, are localized more to the core of the lipoproteins (16). In the exchange of tocopherol there appears to be a difference between the lipoproteins. Although the half-times for VLDL and HDL are the same, the $K_{RBC \rightarrow VLDL}$ is not proportional to the concentrations of VLDL tocopherol when compared with $K_{RBC \rightarrow Pl}$ and $K_{RBC \rightarrow HDL}$. Also, the $K_{VLDL \rightarrow RBC}$ ($0.066\ hr^{-1}$) is only about 60% of the value of $K_{HDL \rightarrow RBC}$ ($0.113\ hr^{-1}$). In all of the different exchanges studied, tocopherol exchange between RBC and VLDL was the only instance in which the calculated flux in vs. flux out were widely divergent, indicating that this exchange does not fit the closed, two-compartment model. One possible explanation for these results is that the VLDL contains two pools of tocopherol, one that exchanges with RBC at the same rate as HDL and one that does not exchange directly with RBC and equilibrates slowly with the first VLDL tocopherol pool. An alternative explanation might be that the isolation procedure and subsequent storage (1–2 wk at 4°C in EDTA buffer) have altered the ability of the VLDL to exchange tocopherol; this seems unlikely because in the same VLDL preparation free cholesterol exchange with RBC is rapid and reaches equilibrium.

Chylomicrons and VLDL from human serum have recently been isolated in narrow size ranges, and their chemical compositions have been determined. Calculations based on these results were consistent with a model having free cholesterol, protein, and phospholipid present in a thin film around an inner core of cholesteryl ester and triglyceride (14). Our results from the studies of cholesterol exchange between VLDL and RBC appear compatible with this model; if free cholesterol is located on the outside of the lipoprotein, its exchange with RBC cholesterol should be rapid and complete. The location of these lipids within the VLDL molecule may determine whether they exchange or do not exchange with RBC, since it is known that the more polar cholesterol and phospholipids do exchange, and the less polar cholesteryl ester and triglyceride, thought to be in the core, do not (41). The distribution of tocopherol between surface and core in VLDL lipoproteins is not known. Assuming that the model for VLDL is correct, a possible explanation for the nonequilibration of [^{3}H]tocopherol between the VLDL and RBC might be that tocopherol is distributed throughout the lipoproteins in such a way that only the portion of tocopherol located on the surface with free cholesterol exchanges at the same rate as HDL tocopherol but the portion in the core of the VLDL along with the less polar cholesteryl esters and triglycerides would not exchange readily with RBC tocopherol. This

would require that the exchange of tocopherol between the core and surface pools of VLDL tocopherol be slow compared with its exchange between RBC and the VLDL surface pool ($t_{1/2}$ 2–3 hr). Since HDL is of much smaller size, core and surface tocopherol may exchange rapidly and appear to be one pool.

The experimental evidence supporting this hypothesis was obtained by disrupting rat plasma VLDL by procedures, described by Zilversmit and others (31, 35–37), that separate the coat and core material of chylomicrons. In comparing the percentage of the total VLDL free cholesterol and tocopherol isolated with the phospholipid–protein portion after disruption, it was found that a greater percentage of free cholesterol is associated with this surface material. It was estimated that roughly two-thirds of the free cholesterol, but only one-third of the VLDL tocopherol, is associated with the phospholipid–protein portion. Similar experiments have not been carried out with HDL because of its greater stability and smaller size. The fact that not all of the free cholesterol is found associated with the phospholipid may not be evidence against the VLDL model of Sata, Havel, and Jones (14) because the methods used here to disrupt the VLDL molecule were harsh and could well have changed the original distribution (e.g., fragments of VLDL containing both coat and core material may have been broken off). A second possibility is that further exchange of the lipids could have taken place during the actual disruption procedures, which are carried out over a period of 4–5 hr. If this were the case, it is possible that the separation of polar and nonpolar VLDL constituents is initially much sharper but that redistribution takes place during the time of disruption, either by transfer or by recombination. In view of these uncertainties, it is emphasized that these techniques yield only qualitative data, indicating that disrupted rat plasma VLDL can be separated into two fractions, one of which contains a much higher proportion of polar compounds. We interpret the observation that free cholesterol and tocopherol are distributed differently between these two fractions as indicating that these molecules have different locations within the intact VLDL. Although it is possible that this difference is the result of the disruption procedure, we consider it unlikely, because several different disruption techniques were used and the distribution pattern of free cholesterol and tocopherol was consistently observed. This difference in distribution was also evident when different amounts of phospholipid and protein were removed from the VLDL (Table 7). If tocopherol were functioning in vivo as a lipid antioxidant (42), all of the VLDL lipids, both polar and nonpolar, would be protected because tocopherol appears to be distributed in proportion to the total amount of lipids.

If core and surface pools of tocopherol in the VLDL do exist, it is not apparent how [^{3}H]tocopherol reaches equilibrium when VLDL and HDL are incubated together. However, it has been reported that the fractional exchange rate for cholesterol between human LDL and HDL is 3–10 times greater than for the same exchange between each lipoprotein and RBC (34). A more rapid exchange of tocopherol or even a different mechanism of exchange may be the basis for the observed equilibration of [^{3}H]tocopherol between rat VLDL and HDL.

In studies on the exchange of free cholesterol between human erythrocytes and isolated plasma lipoproteins, Quarfordt and Hilderman (34) found that the exchange was more rapid between RBC and HDL than between RBC and LDL, as evidenced by a faster approach to equilibrium and a somewhat higher fractional exchange rate ($K_{HDL \rightarrow RBC} > K_{LDL \rightarrow RBC}$). Although these authors did not study cholesterol exchange in human VLDL and the present study did not quantitate the exchange in rat LDL, it is interesting that the in vitro HDL fractional exchange rate ($K_{HDL \rightarrow RBC}$) for the human studies averaged 0.348 hr^{-1} (calculated from data in Ref. 34) while that for the rat is 0.366 hr^{-1}. Although the cholesterol exchange between HDL and RBC may be similar in humans and rats, the total pattern of exchange between plasma lipoproteins and RBC is quite different because of the presence of the large cholesterol pool in human LDL. In humans, the major amount of free cholesterol exchange between RBC and plasma lipoproteins occurs between RBC and LDL, whereas in the rat, the major amount of free cholesterol exchange occurs between RBC and HDL.

The physiological importance of the physical exchange of tocopherol and free cholesterol between lipoproteins and tissues is not known, although cholesterol (3) and tocopherol (43) are rapidly taken up by tissues from serum lipoproteins. Glomset (9) has proposed a scheme for cholesterol transport involving the physical exchange of free cholesterol between the membranes of tissues and plasma lipoproteins (particularly HDL) that is mediated by the action of LCAT. Davies et al. (44) have shown in orotic acid-fed rats, in which plasma lipoprotein concentrations decrease and the liver accumulates large amounts of lipid, that more of an absorbed dose of radioactive tocopherol is found in the liver and less in plasma and peripheral tissues than in control rats. These authors interpret their results as indicating that a normal lipoprotein transport mechanism is necessary for normal utilization of absorbed tocopherol. An alternative explanation might be that tocopherol is in dynamic equilibration between the tissues and lipoproteins so that as the lipid content of the liver increases, so does the tocopherol content. In either case, an exchange of tocopherol between lipoproteins and tissues is required.

In comparing the relative activities of the rat plasma VLDL and HDL, it appears that for cholesterol exchange with RBC, these lipoproteins have similar kinetics, but that HDL in rat plasma exchanges the greatest quantity of cholesterol simply because it contains the greatest quantity

of cholesterol of the lipoproteins. For tocopherol exchange with RBC, it appears that while the rat plasma VLDL and HDL show the same kinetics initially, tocopherol does not completely equilibrate between RBC and VLDL during the study period. This difference between VLDL and HDL may be important in the respective roles of these lipoproteins in tocopherol metabolism in vivo. It is suggested that the location of cholesterol and tocopherol in the lipoproteins is an important factor in the kinetics and extent of exchange of these compounds between lipoproteins and RBC, and lipoproteins and tissues.

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