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Studies on the distribution of tocopherol in human serum lipoproteins*

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SUMMARY

The tocopherol content of human serum proteins, isolated by density gradient and homogeneous density procedures, was determined by the Emmerie-Engel and phosphomolybdic acid methods. Serum tocopherol is found primarily in the lipoproteins. The low density lipoproteins, principally the S_f 3-9 lipoproteins, contain a major part of the total serum tocopherol, while high density lipoproteins contain the remainder. Serum tocopherol increases after oral administration and remains elevated for 24 hours. The increment in serum tocopherol from newly absorbed tocopherol is found first in the chylomicron and S_f 10-400 lipoprotein fractions. A tocopherol peak occurs in these fractions 4 hours after oral administration. This peak is followed by an increment in the tocopherol content of the S_f 3-9 and high density lipoprotein fractions which reaches a maximum plateau in from 8 to 10 hours and remains elevated from 14 to 16 hours. The distribution of newly absorbed tocopherol is discussed in relation to lipid absorption and transport of other lipids by the serum lipoproteins.

The distribution of tocopherol in the human serum lipoproteins has received limited study. There appears to be a controversy as to whether tocopherol is bound to all the serum proteins (1), bound to low density lipoproteins (2), or bound principally to high density lipoproteins (3). In the present study the distribution of tocopherol in the serum proteins was observed before, during, and after the oral administration of tocopherol to humans.

METHODS

Separation of Lipoproteins. Lipoprotein fractions were separated from human serum by the ultracentrifugal flotation procedure of Havel *et al.* (4). Serum was adjusted to a density of 1.21 g. per ml. with a sodium chloride-potassium bromide stock solution and centrifuged from 18 to 22 hours at 100,000 $\times$ *g* in a Spinco Model L ultracentrifuge. The low and high density lipoproteins floated to the top of the centrifuge tube and were then separated from the other serum proteins in the infranatant solution with a tube cutter. In the separation of low and high density lipoproteins, serum was adjusted to a density of 1.063 g. per ml. with a sodium chloride-potassium bromide stock solution and centrifuged from 18 to 22 hours at 100,000 $\times$ *g*.

Low density lipoproteins floated to the top of the centrifuge tube, while high density lipoproteins and other serum proteins remained in the infranatant solution.

Chylomicrons, S_f 10-400, S_f 3-9, and high density lipoproteins were separated by a density gradient ultracentrifugation procedure (5, 6). Five ml. of serum was placed in a 13.5 ml. lusteroid centrifuge tube, overlaid with 4 ml. of 0.15 M sodium chloride,[1] and centrifuged for 30 minutes at 9,300 $\times$ *g* in the Spinco Model L ultracentrifuge. Chylomicrons formed a turbid band at the top of the saline layer. This band was separated from the infranatant serum with a tube cutter. The infranatant serum was then layered over 5.5 ml. of 2.0 M sodium chloride in a second centrifuge tube, overlaid with 0.15 M sodium chloride to fill the tube, and centrifuged at 100,000 $\times$ *g* for 18 to 22 hours. The S_f 10-400 lipoproteins were concentrated in a turbid band at the top of the tube, the S_f 3-9 lipoproteins formed a clear orange-yellow band in the center, while high density lipoproteins and other serum proteins sedimented to the bottom of the tube.

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[1] All salt solutions added in the preparation of lipoproteins contained 0.05 g./l. of the disodium salt of ethylenediaminetetraacetic acid adjusted to pH 7.0 $\pm$ 0.2 with 1 *N* sodium hydroxide (5).

Tocopherol Administration. Two g. of tocopherol[2] was given to young healthy adults with a lipid meal consisting of one-half pint of ice cream and 4 ounces of eggnog. The lipid meal produced an alimentary lipemia. Control experiments were run with the lipid meal alone and with the tocopherol alone. When given without a lipid meal, the dose of tocopherol was reduced to 600 mg. in order to prevent diarrhea. A blood specimen was drawn before and at 2- to 4-hour intervals after the lipid meal. Blood specimens were obtained over a 24-to-48-hour period. Four ml. of serum was used for total serum tocopherol analysis and 5 to 6 ml. for the separation of lipoprotein fractions. Two subjects designated as I and II were used throughout the experimental series for the study of tocopherol absorption and distribution in lipoproteins. Subject I was a 28-year-old healthy male and subject II was a 24-year-old healthy female.

Tocopherol Assay. For the determination of serum tocopherol and its distribution in the serum lipoprotein fractions, the phosphomolybdic acid and Emmerie-Engel (EE) reactions were compared. A modification of the Rosenkrantz procedure (7) was used for the phosphomolybdic acid reaction (PMA). Four ml. of serum or a lipoprotein fraction isolated from 4 ml. of serum was denatured with an equal volume of ethanol and extracted with 15 ml. of isooctane. A 10 ml. aliquot of the isooctane solution was placed in a 50 ml. boiling flask and dried under nitrogen at 40°C in a rotary vacuum evaporator. The residue was first dissolved in 2 ml. of repurified butanol and then 2 ml. of fresh 1.6 per cent phosphomolybdic acid in glacial acetic acid was added. This sample was transferred to a 15 × 150 mm. Pyrex test tube, placed in a 55°C water bath for 15 minutes, and then cooled at room temperature for 30 minutes. Absorbance was determined at 725 mμ. with a Beckman B spectrophotometer. Since carotenoids interfere in the PMA reaction, the carotenoid content of the isooctane extract was estimated from the absorbance of an isooctane aliquot at 450 mμ. Standards of α-tocopherol, the major tocopherol constituent of serum (8), and β-carotene were used in each set of determinations.

Phospholipids and cholesterol interfere in the PMA reaction. Lecithin, isolated from egg yolk by silicic acid chromatography, produces a white precipitate with phosphomolybdic acid upon cooling. Turbid samples were filtered through a pressure filter system consisting of a 10 ml. glass syringe containing two layers of Whatman No. 1 and two layers of Whatman No. 42 filter paper. Phosphomolybdic acid reacts with free cholesterol at incubation temperatures above 60°C and enhances the absorbance at 725 mμ. The free cholesterol in serum inhibits the PMA-tocopherol color reaction by 4 to 5 per cent at incubation temperatures of 55°C. This cholesterol inhibition of the PMA reaction was corrected by the use of tocopherol internal standards. Cholesterol palmitate (recrystallized: m.p. 77°C) had no effect on the PMA reaction.

The PMA reaction measures total tocopherol. When α-tocopherol quinone, prepared by the silver nitrate oxidation procedure of Cuthbertson *et al.* (9), was assayed with the modified PMA procedure, 69 per cent of the color expected from α-tocopherol was obtained. This corresponds to the value obtained for α-tocopherol quinone in the unmodified Rosenkrantz procedure (7).

The ferric chloride-α,α-bipyridine reaction as modified by Farber *et al.* (10) was used for the EE reaction. Four ml. of serum or a lipoprotein fraction was denatured with an equal volume of ethanol and extracted with 4 ml. of xylene for 10 minutes in a mechanical shaker. The emulsion was broken by centrifugation and 3 ml. of the xylene extract transferred to a 19 mm. Coleman cuvette. Three ml. of 0.1 per cent α,α-bipyridine in *n*-propanol was added and the carotenoid absorbance determined at 440 mμ. with a Coleman Nephelo-Colorimeter. Then 0.5 ml. of 0.1 per cent ferric chloride was added, the sample shaken, and read at 525 mμ. after a 2-minute interval. The color developed with pure α-tocopherol was stable from 30 seconds to 10 minutes; however, the color reaction with β-carotene increased with time.

Chemical Analysis. Lipoprotein fractions were placed in volumetric flasks and the lipid extracted with alcohol-ether (3:1). Cholesterol was determined by the method of Abell *et al.* (11). Lipid phosphorus was determined by the method of Lowry *et al.* (12) and multiplied by 25 to estimate phospholipid. Fatty acid ester equivalents were determined by the method of Stern and Shapiro (13).

RESULTS

The Distribution of Tocopherol in Serum Proteins. The fasting serum tocopherol levels are higher when measured by the PMA than by the EE assay method (Tables 1 and 2). Since the PMA reaction estimates tocopherol quinone as well as free tocopherol, these values are higher and are consistent with the report (14) that approximately 30 per cent of serum tocopherol is in the quinone form. No significant difference

[2] Eprolin®, lipid soluble tocopherol, Eli Lilly Co., Indianapolis, Ind.

TABLE 1. TOCOPHEROL DISTRIBUTION IN LOW AND HIGH DENSITY LIPOPROTEIN FRACTIONS OBTAINED BY ULTRACENTRIFUGAL FLOTATION *

Fraction	A	B	C
	Tocopherol in μg./ml. and per cent (*italics*) of total recovered		
Serum	13.8	9.2	23.3
Low and high density lipoproteins (D < 1.21)	13.3 *83*	5.8 *87*	21.7 *94*
Other serum proteins	2.7 *17*	0.9 *13*	1.4 *6*
Total recovered	16.0	6.7	23.1
Percentage recovered	*116*	*72*	*99*
Low density (D < 1.063) lipoproteins	9.6 *66*	5.9 *63*	16.8 *71*
High density lipoproteins and other serum proteins	5.0 *34*	3.5 *37*	7.4 *29*
Total recovered	14.6	9.4	24.2
Percentage recovered	*106*	*102*	*104*

* Ultracentrifugal flotation procedure of Havel *et al.* (4).
A. Subject I, fasting serum, PMA analysis.
B. Subject II, fasting serum, EE analysis.
C. Subject II, 10 hours after test meal with tocopherol, EE analysis.

was found between the tocopherol content of serum and plasma by either assay method.

Serum was fractionated into low and high density lipoproteins by the homogeneous density method and these fractions were analyzed for their tocopherol content by the PMA and EE reactions (Table 1). Over 83 per cent of the tocopherol was found in the serum lipoprotein fractions. The relative amount of tocopherol associated with other serum proteins decreased from 13 per cent to 6 per cent after the oral administration of tocopherol (Table 1). From 63 to 71 per cent of the serum tocopherol was carried by the chylomicrons and low density lipoproteins.

Serum was fractionated into chylomicrons, S_f 10-400, S_f 3-9, and high density lipoproteins by the density gradient method and these fractions were analyzed for their tocopherol content by the PMA and EE reactions (Table 2). The chylomicrons and low density lipoproteins, isolated by the density gradient procedure, contained from 65 to 72 per cent of the serum tocopherol. This tocopherol distribution corresponds to the tocopherol distribution obtained in the separation of low density lipoproteins by the homogeneous density centrifugation procedure (Table 1). The S_f 3-9 lipoprotein fraction contained from 41 to 58 per cent of the serum tocopherol. Tocopherol activities were also determined on lipid extracts from the lipoproteins of subject I by the carotenoid stability test (15). This procedure showed that 69 per cent of the total tocopherol activity was in the S_f 3-9 lipoprotein fraction. Since the PMA and EE reactions showed a similar tocopherol distribution, no difference was detected between the relative content of tocopherol and tocopherol quinone in the serum lipoprotein fractions.

Tocopherol analyses by the PMA reaction on duplicate lipoprotein fractions varied ± 0.1 μg. per ml. to ± 0.4 μg. per ml. from the average of the two determinations. Tocopherol analyses by the EE reaction on duplicate lipoprotein fractions varied ± 0.2 μg. per ml. to ± 0.4 μg. per ml. from the average of the two

TABLE 2. Tocopherol Distribution in Chylomicron, S_f 10-400, S_f 3-9, and High Density Lipoprotein Fractions Obtained by Ultracentrifugal Flotation *

Fraction	A	B	C	D	E
	Tocopherol in μg./ml. and per cent (*italics*) of total recovered				
Serum	12.2	9.1	7.35	6.34	13.1 ± 2.7
Chylomicron	0.14 *1.3*	0.47 *5.2*	0 *0*	0.12 *1.8*	0.51 ± 0.34 *4.0 ± 2.7*
S_f 10-400	1.09 *11*	1.67 *18*	0.67 *9.3*	0.61 *9.8*	1.97 ± 0.62 *15.5 ± 4.9*
S_f 3-9	5.34 *53*	4.38 *48*	4.18 *58*	3.53 *57*	5.96 ± 0.75 *47.0 ± 5.9*
High density lipoproteins and other serum proteins	3.54 *35*	2.58 *28*	2.38 *33*	1.95 *32*	4.24 ± 0.48 *33.4 ± 3.8*
Total recovered	10.1	9.1	7.23	6.21	12.7 ± 1.2
Percentage recovered	*83*	*100*	*98*	*98*	*97 ± 8.7*

* Density gradient ultracentrifugal flotation procedure (5, 6).
A. Subject I, fasting serum, PMA analysis.
B. Subject I, fasting serum, EE analysis.
C. Subject II, fasting serum, PMA analysis.
D. Subject II, fasting serum, EE analysis.
E. 10 subjects, nonfasting serum, PMA analysis, mean ± standard deviation.

determinations. The range in tocopherol content represents the error from both the tocopherol assay and the lipoprotein fractionation.

Tocopherol Distribution During the Active Absorption of Tocopherol. The concentration of serum tocopherol after oral tocopherol administration to subjects I and II is shown in Figure 1. When 2.0 g. of tocopherol was administered to subject II with a lipid meal, the increment in serum tocopherol reached one peak in 6 hours and a second peak in 12 hours. The second peak occurred 3.5 hours after the subject had eaten a regular meal and may represent absorption of unabsorbed tocopherol or reabsorption of biliary excreted tocopherol (16). When 600 mg. of tocopherol was administered to subject I without a lipid meal, a smaller but significant increment in serum tocopherol was obtained. Tocopherol was administered without a lipid meal in order to demonstrate an elevation in serum tocopherol without the parallel elevation of serum lipids, which was verified by phospholipid analysis of the lipoprotein fractions. Serum tocopherol remained elevated for the duration of the test period in both experiments in which supplementary tocopherol was given. When a lipid meal without supplementary tocopherol was given to subject I, no rise in serum tocopherol occurred (Fig. 1).

Serum lipoprotein fractions were separated by the density gradient ultracentrifugation procedure, and tocopherol increments in the lipoprotein fractions were measured at stated time intervals for the duration of the test period. The chylomicron and S_f 10-400 lipoprotein fractions showed a peak tocopherol increment 4 hours after tocopherol administration (Figs. 2 and 3). When 2.0 g. of tocopherol was administered to the subject, a second peak occurred in the chylomicron and S_f 10-400 lipoprotein fractions 3 to 4 hours after the subject had eaten a second meal (Fig. 2). A single peak in the tocopherol increment was found for both the chylomicron and S_f 10-400 lipoprotein fractions when 600 mg. of tocopherol was administered to a subject (Fig. 3). The tocopherol content of the S_f 3-9 lipoprotein fraction reached a maximum from 6 to 10 hours after the test meal and maintained a plateau for another 12 hours (Figs. 2 and 3). The tocopherol increment in the high density lipoprotein fraction follows a pattern similar to that in the S_f 3-9 lipo-

protein fraction, although the increment was less in the high density lipoprotein fraction.

Table 3 shows the tocopherol distribution in the lipoproteins following a lipid meal without supplementary tocopherol. One pint of ice cream, a test meal that produces lipemia without elevating serum tocopherol, was given to subject I. Blood specimens were obtained at 0, 2, 4, and 8 hours, and serum lipoprotein fractions isolated. The lipoprotein fractions were analyzed for tocopherol (EE reaction), fatty acid ester equivalents, cholesterol, and phospholipid. The lipid meal produced visible lipemia and a significant increase in the concentration of the fatty acid esters in the chylomicron and S_f 10-400 lipoprotein fractions, without altering the tocopherol content of these two fractions. The lipid and tocopherol concentrations of the S_f 3-9 and high density lipoprotein fractions were not altered by the lipid meal.

TABLE 3. COMPOSITION OF THE CHYLOMICRON AND THE S_f 10-400 LIPOPROTEIN FRACTIONS AFTER A LIPID MEAL (ICE CREAM) WITHOUT SUPPLEMENTARY TOCOPHEROL *

Fraction and Time in Hours	Tocopherol	Fatty Acid Ester	Ratio †
	μg./ml.	*meq./100 ml.*	
Chylomicron			
0	0.30	0.004	17.2
2	0.33	0.010	7.6
4	0.33	0.070	1.1
8	0.30	0.030	2.3
S_f 10-400 lipoproteins			
0	1.44	0.11	3.0
2	1.48	0.18	1.9
4	1.48	0.21	1.6
8	1.07	0.15	1.6

* Density gradient ultracentrifugal flotation procedure (5, 6).
† Tocopherol (μmole)/fatty acid ester (meq.).

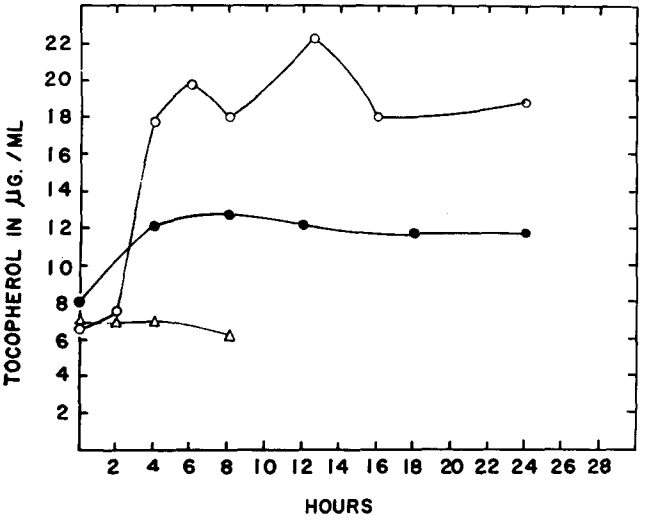

FIG. 1. The concentration of tocopherol (EE reaction) in serum after oral administration. Tocopherol, 2.0 g., administered to subject II with a lipid meal (o). Tocopherol, 600 mg., administered to subject I without a lipid meal (●). Lipid meal without supplementary tocopherol administered to subject I (Δ).

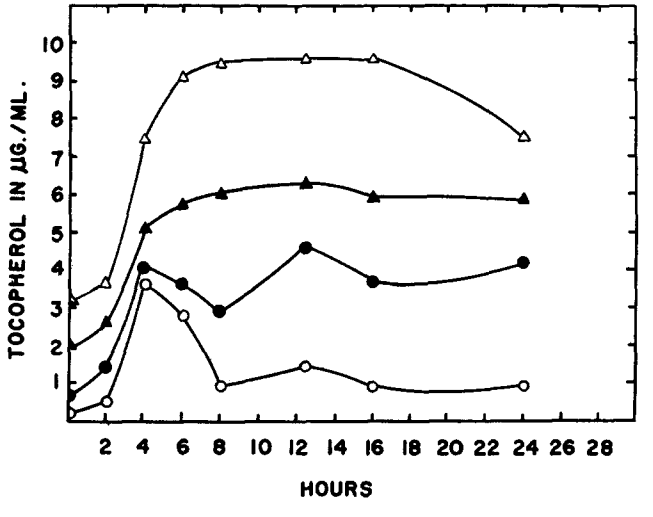

FIG. 2. The tocopherol content (EE reaction) in μg./ml. serum for lipoprotein fractions separated by density gradient flotation from subject II after the oral administration of 2.0 g. tocopherol with a lipid meal: chylomicrons (o), S_f 10-400 lipoproteins (●), S_f 3-9 lipoproteins (Δ), high density lipoproteins and other serum proteins (▲).

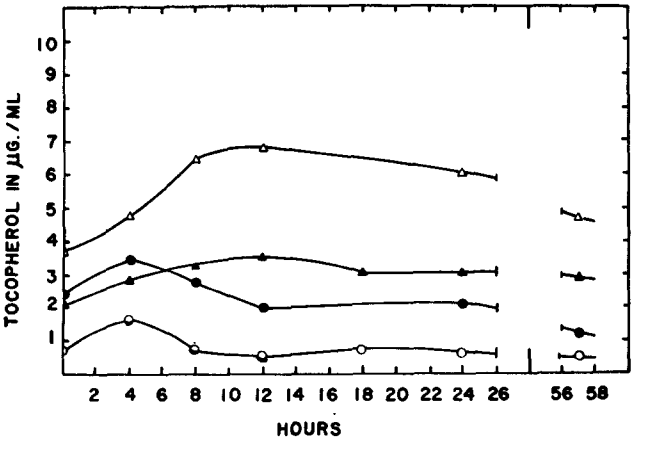

FIG. 3. The tocopherol content (EE reaction) in μg./ml. serum for lipoprotein fractions separated by density gradient flotation from subject I after the oral administration of 600 mg. tocopherol without a lipid meal: chylomicrons (o), S_f 10-400 lipoproteins (●), S_f 3-9 lipoproteins (Δ), high density lipoproteins and other serum proteins (▲).

Tocopherol Exchange Between Lipoproteins in Vitro. The tocopherol equilibration between low density and high density lipoprotein fractions was studied by tocopherol exchange *in vitro*. Blood was obtained from subject II before a test meal and 10 hours after the administration of 1.2 g. of tocopherol with a test meal. Duplicate low and high density lipoprotein fractions were separated by centrifugation at density 1.063. One lipoprotein fraction was used for a tocopherol exchange study; the other lipoprotein fraction was used for tocopherol analysis. A low density lipoprotein fraction from fasting serum was mixed with a high density lipoprotein fraction from the tocopherol-rich serum (Mixture A), and a low density lipoprotein fraction from the tocopherol-rich serum was mixed with a high density lipoprotein fraction from fasting serum (Mixture B). These lipoprotein mixtures were equilibrated at 37°C under nitrogen by agitation for 6 hours. Lipoprotein fractions were then reisolated by centrifugation at density 1.063 and analyzed for tocopherol. The results are summarized in Table 4. Tocopherol exchanged between the low and high density lipoprotein fractions. The final tocopherol distributions, 57 to 61 per cent in low density lipoproteins and 39 to 43 per cent in high density lipoproteins, were similar for the two mixtures. Furthermore, the percentage distribution of tocopherol obtained by equilibration *in vitro* was similar to the tocopherol distribution found in serum.

DISCUSSION

Essentially all serum tocopherol is found in the serum lipoprotein fractions. The small amount of apparent tocopherol found in the other serum proteins (density > 1.21) may be due to either vitamin A alcohol (6), which reacts in the analytical procedures, or to the incomplete separation of high density lipoproteins during the isolation procedure (4). If tocopherol were associated with other serum proteins, their tocopherol content should increase with increased levels of serum tocopherol; but this did not occur. Although albumin has been shown to combine with tocopherol *in vitro* (17), it does not appear to participate in tocopherol transport *in vivo*. Other workers did not detect radioactivity in the serum albumin fraction isolated from rats fed C^{14}-tocopherol (18).

In the fasting state the low density lipoproteins, principally the S_f 3-9, contain a major part of the serum tocopherol. This observation is in contradiction to an earlier observation (3) that the high density lipoproteins are the principal carriers of tocopherol. Independent studies have shown that elevated tocopherol levels and elevated low density lipoprotein levels occur in hypercholesterolemic disease states such as myxedema, idiopathic hypercholesterolemia, diabetes mellitus, xanthomatosis, coronary occlusion, cerebral thrombosis, and hypertensive cardiovascular disease (16, 19, 20, 21). During pregnancy there is a parallel rise in tocopherol (22) and low density lipoprotein levels (23), which reaches a peak at the third trimester.

Following oral administration of tocopherol with a lipid meal, the concentration of tocopherol in the chylomicron and S_f 10-400 lipoprotein fraction reaches a maximum value in 3 to 4 hours, a time at which the lipid content of these two fractions is also at a maximum (24, 25, 26). In control experiments, the separate administration of either tocopherol or lipid produces an independent elevation of either tocopherol or lipid, respectively, in the chylomicron and S_f 10-400 lipoprotein fractions. Thus the initial elevation of tocopherol in these fractions after tocopherol absorption represents newly absorbed tocopherol and not the exchange of tocopherol between the different lipoproteins, whose relative concentration is changing as a result of simultaneous lipid absorption.

The first phase of tocopherol transport by the chylomicrons and S_f 10-400 lipoproteins is followed by a second phase in which newly absorbed tocopherol shows a prolonged elevation in the S_f 3-9 and high density lipoproteins. The prolonged elevation is a reflection of the long half life of these latter fractions (27) as contrasted to the very short half life of the chylomicron (28) and S_f 10-400 fractions (27). It is noteworthy that the tocopherol increment in the S_f 3-9 lipoprotein and the high density lipoprotein fractions reaches a maximum in 8 to 10 hours, a time when the chylomicron and S_f 10-400 lipoprotein fractions are approaching basal levels. It appears that tocopherol is transferred to the S_f 3-9 and high density lipoproteins from the chylomicrons and S_f 10-400 lipoproteins. Metabolic sequences for these reactions are not defined completely; however, the conversion of S_f 10-400 lipoproteins to S_f 3-9 lipoproteins *in vivo* has been demonstrated (27). High density lipoproteins and chylomicrons may also be related in their metabolism since these fractions have a similar protein moiety (29, 30) which is distinct immunologically from the low density lipoproteins (31). Tocopherol equilibration studies *in vitro* and the parallel course of tocopherol increments in the S_f 3-9 and high density lipoprotein fractions both suggest that tocopherol exchanges between low density and high density lipoproteins in a manner similar to cholesterol (28) and phospholipid (32). Thus the second phase of tocopherol transport, a to-

TABLE 4. TOCOPHEROL EXCHANGE, *In Vitro*, BETWEEN LOW DENSITY AND HIGH DENSITY LIPOPROTEIN FRACTIONS

Sample	Lipoprotein Tocopherol in μg./ml. and per cent (*italics*) of total recovered	
	Low Density D < 1.063	High Density D > 1.063
Serum fasting	5.9	3.5
	63	*37*
Serum 10 hours after test meal	17.2	7.0
	71	*29*
Mixture A		
Pre-equilibration	5.9	7.0
	*46**	*54**
Postequilibration (6 hours)	7.3	5.5
	57	*43*
Mixture B		
Pre-equilibration	17.2	3.5
	*83**	*17**
Postequilibration (6 hours)	12.5	8.0
	61	*39*

* Calculated for mixture.

Mixture A: Low density lipoproteins from fasting serum and high density lipoproteins from tocopherol-rich serum.

Mixture B: Low density lipoproteins from tocopherol-rich serum and high density lipoproteins from fasting serum.

copherol increment in the S_f 3-9 and high density lipoproteins, represents accumulation of tocopherol in the two major lipoprotein fractions of serum, together with exchange of tocopherol between these lipoproteins and a final tocopherol distribution similar to their total lipid content (33).

Total serum tocopherol shows an absorption increment of long duration because it enters into both the first and second phases of lipid transport by serum lipoproteins. Lipids such as triglycerides (25, 28) and vitamin A esters (6), which enter only the first phase of lipid transport, demonstrate an absorption increment of short duration. Triglycerides (34) and vitamin A esters (35) are both hydrolyzed after their initial concentration in the chylomicron and S_f 10-400 lipoprotein fractions and their hydrolysis products are subsequently not lipoprotein bound; whereas tocopherol is transferred to the S_f 3-9 and high density lipoproteins, which have a longer half life than do the chylomicrons and S_f 10-400 lipoproteins.

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