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Kinetics of *rac*-1-Oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol Transfer between High Density Lipoproteins†

Steven C. Charlton, Kong-Yi Hong, and Louis C. Smith*,‡

ABSTRACT: The mechanism of transfer of diglyceride between high density lipoproteins (HDL) was investigated with a pyrene-containing analogue whose fluorescent properties depend on the microscopic concentration in the lipoprotein. Transfer rates were first order, rapid (3.5 s^{-1}), and invariant over a 100-fold range of HDL concentration and over a 10-fold range of *rac*-1-oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol concentrations. Similar behavior of the probe was observed with HDL_3, although the rate was 40% slower. These results support a mechanism in which rate-limiting dissociation of the diglyceride analogue from one HDL particle into the aqueous phase precedes rapid diffusion and subsequent uptake by another such particle.

The equilibration of the phospholipids (Illingworth & Portman, 1972a,b), triglycerides (Quarfordt et al., 1971), and cholesterol (Bruckdorfer & Green, 1967; Basford et al., 1971) among various lipoproteins and tissue pools is a well-documented process. However, the time scale and the mechanism by which lipid transfer occurs in the absence of specific exchange proteins (Butler & Thompson, 1975; Wirtz et al., 1972) are not well established. Previous studies had poor time resolution and were complicated by the need to separate products of exchange. To circumvent these problems, we have used a probe containing pyrene, a molecule with fluorescence properties which depend on the local concentration of the compound.

Dilute solutions of pyrene exhibit a fluorescence from the lowest excited singlet state with a maximum at 390 nm. This is termed monomer fluorescence. At higher pyrene concen-

† From the Division of Atherosclerosis and Lipoprotein Research, Departments of Medicine and Biochemistry, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030. *Received September 12, 1977; revised manuscript received May 4, 1978.* Support for the research was provided by grants from the Robert A. Welch Foundation Q-343, The American Heart Association 73-778, U.S. Public Health Service HL-15648 and HL-17269, and NIHLB Lipid Research Clinic Contract 71-2156.

‡ Established Investigator of the American Heart Association. Address correspondence to this author at: Baylor College of Medicine, Methodist Mail Station—A601, Houston, Texas 77030.

0006-2960/78/0417-3304$01.00/0

trations, an *excited dimer* (excimer) is produced by the transient association of an excited singlet state molecule with a ground state pyrene molecule. As the pyrene concentration increases, excimer emission at 470 nm increases at the expense of the monomer emission, a special case of fluorescence quenching. The ratio of these intensities, *E/M*,[1] is directly proportional to the pyrene concentration (Pownall & Smith, 1973). This property of pyrene and its derivatives can be used to monitor their transfer if a net concentration change occurs. The same strategy has been successfully applied to pyrene transfer between high density lipoproteins, HDL (Charlton et al., 1976a), and to transfer of 10-(3-pyrenyl)decanoic acid between phosphatidylcholine vesicles (Sengupta et al., 1976).

In this paper, we present experiments to show that the probe, a fluorescent diglyceride analogue, *rac*-1-oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol, DG (Figure 1), is confined to the lipoprotein and that the rapid rate of transfer of this neutral lipid is invariant under a variety of conditions. A mechanism consistent with the data is also presented. A preliminary report has appeared (Charlton et al., 1976b).

Experimental Procedure

HDL were prepared from fresh, unfrozen, single donor human plasma by ultracentrifugal flotation. The solution density was adjusted to 1.21 g mL^{-1} with solid KBr and centrifuged at 10 °C for 44 h at 45 000 rpm in a 60 Ti rotor (Havel et al., 1955). The lipoproteins were isolated by tube slicing, dialyzed against 0.15 M NaCl containing 0.02 M Tris-Cl, pH 7.4, 1 mM EDTA, and 1 mM NaN_3 before chromatography on a 6 × 60 cm column of Bio-Gel A-5m (Rudel et al., 1974) equilibrated with this buffer. HDL_3, density range 1.125 to 1.25 g mL^{-1}, were prepared by sequential ultracentrifugation of HDL at 10 °C (Scanu, 1966). The composition of HDL was assumed to be 50% by weight of protein (Skipski, 1972), the amount of which was determined by the Lowry method (Lowry et al., 1951). Before use, HDL were dialyzed overnight against at least 100 vol of the standard buffer, 0.05 M Tris-Cl, pH 7.4, containing 0.15 M NaCl.

rac-1-Oleylglycerol (350 mg) (Baumann & Mangold, 1964) was acylated in 25 mL of benzene and 1 mL of pyridine with 4-(3-pyrenyl)butanoyl chloride, prepared in situ from 600 mg of the acid and an excess of oxalyl chloride, which was removed in vacuo before the reaction solvent was added. Mono- and diacylated compounds were extracted from the dried residue by $CHCl_3$ extraction and separated by preparative thin-layer chromatography ($CHCl_3$:acetone, 96:4 v/v) on silica gel G impregnated with boric acid (Thomas et al., 1965). Approximately 260 mg of the monoester and 130 mg of the diester were obtained. The yields of the products were estimated by absorbance. The diester (100 mg) was emulsified with 5 mL of 1% poly(vinyl alcohol) (Bier, 1955) containing 0.1 M Tris, pH 8.1, and 40 mg of fatty acid poor albumin for hydrolysis of the primary ester by 50 μg of pancreatic lipase (Worthington, Code:PLI). The progress of the reaction was monitored by TLC and stopped with acetone after 20 min at 37 °C. The organic phase was dried and the desired product was purified initially by preparative thin-layer chromatography as before and finally by reversed phase chromatography on Waters C_{18} Bondpak with methanol:water (80/20 v/v) as solvent. Recycling ten times showed only a single component whose mass

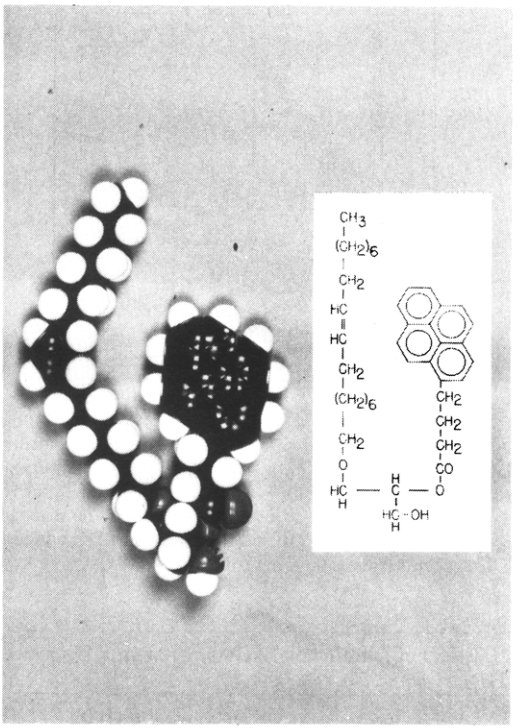

FIGURE 1: Space-filling model of *rac*-1-oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol, DG.

spectra were consistent with the assigned structure. Electron impact mass spectra were obtained using an LKB Model 9000 single focusing instrument with a direct inlet probe; ionizing energy was 70 eV. Mass spectrum: *m/e* (relative abundance) 344 (46.6%); 281 (0.2%); 271 (8.3%); 228 (31%); 215 (100%); 202 (4.1%); 108 (12.2%); 95 (4.1%); 69 (1%); 58 (2.3%); 57 (1.2%); 56 (1.8%); 41 (4.7%); 43 (1.8%); 29 (3.9%); 27 (1.8%). Chemical ionization mass spectra using NH_3 were obtained with a Hewlet Packard 5980A integrated GLC–MS computer. The compound gave a symmetrical surge peak after 12 min of chromatography on 1% OV-17 on Chromosorb W in a 1.7 m × 2 mm glass column. Flow rate was 17 mL min^{-1} N_2; the temperature increase was programmed between 140 and 300 at 8 °C min^{-1}. To obtain mass spectra, the column was maintained at 240 °C. The spectrometer was tuned with PFBA in the electron impact mode at approximately 60 eV. The spectrometer was then directly switched to the ammonia chemical ionization mode and tuned (by varying the ammonia concentration) against peracetylated glucononitrile until a maximum percentage of M + 18 ion was observed, approximately 70% of total *m/e*. The ionization chamber pressure was approximately 50 Torr ammonia. Mass spectrum: *m/e* (relative abundance) 373 (1.7%); 362 (6.2%); 345 (51.1%); 344 (33.7%); 271 (100%); 228 (9.6%); 215 (29.2%). No acyl migration occurred during isolation or purification.

A molar extinction coefficient of 50 000 M^{-1} cm^{-1} at 341 nm was assumed by analogy to pyrene (Pownall & Smith, 1973). HDL were labeled by injection of a small volume of a 10 mg mL^{-1} ethanolic solution directly into the lipoprotein solution as indicated in the tables and figures.

Fluorescence emission spectra were recorded on an Aminco-Bowman spectrofluorimeter with excitation at 328 nm. Calibrated gain controls were used to magnify the signal in the excimer region near 470 nm. Kinetic measurements were obtained on a Durrum-Gibson stopped-flow spectrometer operated in the fluorescence mode. The sample was excited by either a stabilized Liconix Model 4110 He–Cd laser (2 mW at

[1] Abbreviations are: *E/M*, the ratio of relative fluorescence intensity at 470 nm to that at 390 nm; *M*, monomer fluorescence; *E*, excimer fluorescence; HDL, high density lipoproteins; DG, diglyceride ether, *rac*-1-oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol; Me_2SO, dimethyl sulfoxide; PFBA, perfluorotributylamine.

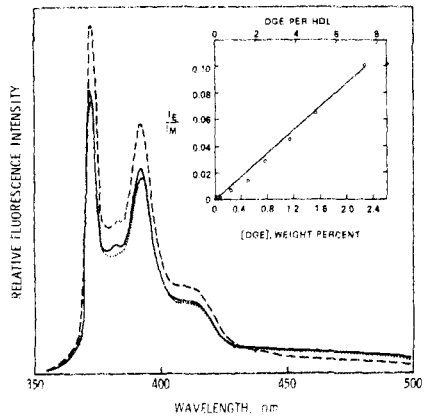

FIGURE 2: Effect of dilution with HDL or buffer on the spectra of DG in HDL. HDL, 0.66 mg mL^{-1} containing 2% DG (—); dilution with an equal volume of HDL solution (- - -); dilution with an equal volume of buffer (• • •). The spectra are adjusted for dilution. The inset shows the linear relationship between E/M and the amount of DG added to HDL. The HDL concentration is 0.66 mg mL^{-1}.

TABLE I: *Effect of Dilution on* Excimer:Monomer Fluorescence of DG in HDL.

HDL concn (μg mL^{-1})	DG concn in HDL: 3% E/M	1% E/M
1000	0.286	0.077
100	0.271	0.070
10	0.243	0.072
1	0.096	0.037

325 nm) or a xenon lamp (331 nm, 1 mm slits). Monomer fluorescence was isolated with a 390-nm interference filter (9.85-nm half band width); excimer fluorescence with a Corning 3-72 filter, transmitting wavelengths longer than 440 nm. Kinetic data were stored in a Biomation Model 805 Waveform recorder. The output was passed through a log amplifier to give an analogue semilog plot from which reaction rates were obtained. All experiments were conducted at 20 °C in the standard buffer unless noted otherwise. Concentrations of DG are given as the percentage weight of the HDL protein.

Results

Mixing a solution of HDL containing DG with an equal quantity of HDL reduced E/M by one-half (Figure 2). A control mixture substituting buffer for HDL produced no ratio change at these concentrations of lipoprotein. To show the probe was confined to HDL, lipoprotein solutions either 1 or 3% DG were sequentially diluted (Table I). The E/M was essentially constant at HDL concentrations between 1 mg mL^{-1} and 10 μg mL^{-1} but decreased precipitously at lower HDL concentrations (10 to 1 μg mL^{-1}). This latter decrease apparently resulted from partitioning of the probe from the lipoprotein into the aqueous phase. The solubility of DG in buffer was estimated by sequential dilution of a 2.5×10^{-5} M water solution prepared by injection into water of 100 μL of 2.5 mM DG in ethanol. As this solution was diluted serially, the initial E/M ratio of 35 decreased to 3.5 (2.5×10^{-6} M), 0.58 (2.5×10^{-7} M), 0.15 (2.5×10^{-8} M) and finally at 2.5×10^{-9} M, no excimer fluorescence was detectable. Consequently, the solubility is less than 2.5×10^{-8} M.

The effect of the amount of diglyceride analogue added to the HDL solution on E/M is shown in the inset of Figure 2. The linear relationship was identical to that found in homogeneous solvents (Pownall & Smith, 1973). Taken together, these observations suggest that the ratio, E/M, is a reliable measure of DG concentration in HDL. Thus, when HDL were added to DG containing HDL, transfer occurred to equalize the microscopic concentration of the probe between the donor and the acceptor lipoprotein (Figure 2). Similar behavior of pyrene has been previously described in this lipoprotein system (Charlton et al., 1976a).

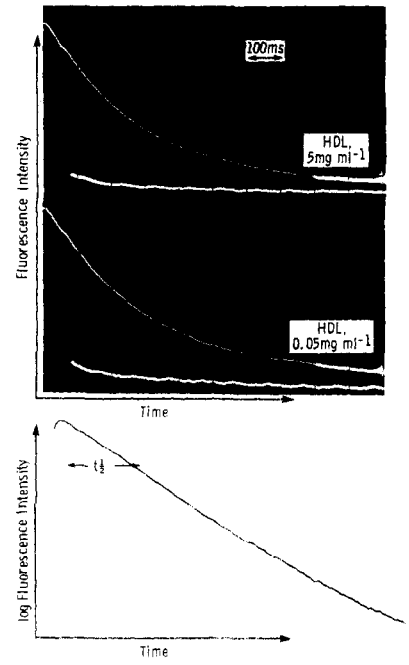

FIGURE 3: Time course of fluorescence changes on mixing. Decrease in excimer fluorescence after mixing either 5 or 0.05 mg mL^{-1} HDL solution with an equal concentration of HDL containing 1% DG. The trace at the bottom of the two upper panels is a continuation of the more extensive initial decrease in excimer fluorescence. The bottom panel is a typical analogue semilog plot of the decrease in excimer fluorescence.

The kinetics of transfer of DG between labeled and unlabeled HDL were investigated on a stopped-flow instrument equipped to monitor fluorescence (Figure 3). In a typical transfer experiment, the fluorescence at 470 nm decreased at the same rate as fluorescence increased at 390 nm. Electrophoretic mobility in agarose of HDL (Noble, 1968) which had been mixed in the stopped-flow unit was identical with that of untreated HDL, indicating the structure integrity of the HDL had been maintained through the mixing process.

The changes in the amplitudes of the excimer fluorescence produced by mixing labeled and unlabeled HDL in the stopped-flow unit were about 35% less than those measured on an Aminco-Bowman spectrofluorimeter. This discrepancy was probably due to the superior spectral resolution and the greatly reduced scattered light in the spectrofluorimeter. Changes in static monomer fluorescence were much smaller and were difficult to quantitate. However, the stopped-flow changes account for most, if not all, of the static reaction. Typically, at least 80% of the amplitude was accounted for by a single exponential decay.

The rate of transfer of DG between lipoproteins was independent of both lipoprotein concentration and the amount of probe incorporated into the lipoprotein (Table II and Figure 3). Even when the lipoprotein concentration was varied by a factor of 100 and the DG to HDL ratio increased by a factor of 10, little or no change in the rate of fluorescence decay was observed. Analogous behavior was exhibited by HDL_3 (Table

TABLE II: Effect of Concentration on the Rate of DG Transfer between HDL.

concn				
HDL[a] (mg mL^{-1})	DG (wt %)	rate at 390 nm (s^{-1})		rate at 470 nm (s^{-1})
4	1	3.73		3.55
0.8	1	3.69		3.54
0.4	1	4.18		3.43
0.04	1	3.65		3.63
0.8	3	3.47		3.45
0.8	0.3	3.79		3.54
		3.75	av	3.52

[a] HDL solutions of equal concentration, one of which contained DG, were mixed as described in Experimental Procedure.

TABLE III: Effect of Concentration on the Rate of DG Transfer between Equal Concentrations of HDL or HDL_3.

	HDL		HDL_3	
lipoprotein concn (mg mL^{-1})	DG (wt %)	rate[a] (s^{-1})	DG (wt %)	rate[a] (s^{-1})
10			1	3.50
1	3	4.71	3	3.77
1	1	5.21	1	3.35
1	0.3	5.35	0.3	3.52
0.1	3	4.75	1	3.32
0.024	3	4.44		
	av	4.87	av	3.49

[a] The temperature was 25 °C.

III) except that the rates were 40% slower than those of HDL.

The experiments indicated that the rate-limiting step is independent of reactant concentration. A possible mechanism is the rate-limiting dissociation of DG into aqueous solution with subsequent rapid diffusion and uptake by HDL. This mechanism could be tested. If conditions are chosen such that dilution of an HDL-DG solution with a large volume of buffer causes an appreciable partitioning of fluorophore into the aqueous region, the rate observed under these experimental conditions should be the same as that observed for transfer between HDL. Repeated attempts to do this experiment were unsuccessful. Because of the very unfavorable distribution coefficient of DG between HDL and buffer, the extremely low concentration of labeled lipoprotein required for the experiment gave such a low fluorescence signal compared with the background signal that no meaningful data were obtained. Increasing the aqueous solubility of DG by the addition of 5% (v/v) Me_2SO to all solutions allows a higher concentration of HDL to be used in the transfer experiments. In Table IV the transfer rates obtained when 1 volume of fluorescently labeled HDL was mixed with 14 volumes of HDL solution or of buffer alone are shown. All solutions contained Me_2SO and the rates were all about 45% faster than in the absence of organic solvent. However, the same rate was found for the transfer from HDL to HDL or from HDL to the solution when no acceptor lipoprotein was present.

The activation energy of the transfer process over the temperature range 10–50 °C was measured. On the basis of the data shown in Figure 4, there appeared to be two distinct processes with E_a of 16.6 and 11.6 kcal mol^{-1}, above and below 36 °C, respectively. HDL solutions which had previously been heated at 50 °C and subsequently cooled to 35 °C showed marked hysteresis. The rate of transfer of DG was 40% faster than that observed with the unheated controls. Presumably, at the higher temperatures, irreversible changes in lipoprotein structure occurred.

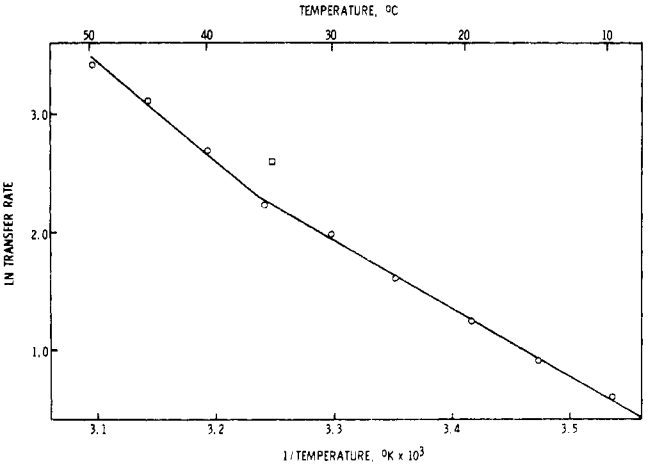

FIGURE 4: Arrhenius plot for transfer rates as a function of increasing temperature. The open square is the value obtained with a solution previously heated to 50 °C and then cooled to 35 °C before the rate of DG transfer was determined. The activation energy is 11.6 kcal mol^{-1} below 36 °C and 16.6 kcal mol^{-1} above.

TABLE IV: Kinetics of Dilution of DG Labeled HDL into Buffer Containing 5% Me_2SO.

lipoprotein concn (mg mL^{-1})[a]			
HDL containing 3% (w/w) DG (1 volume)	HDL (14 volumes)		rate
0.29	1.44		6.9
0.29	0.29		6.8
0.29	0.29		7.6[b]
0.29	0		no change
0.029	1.44		7.0
0.029	0.29		7.3
0.029	0.029		6.9[b]
0.029	0		6.6
		av	7.0

[a] All solutions contained 5% (v/v) Me_2SO. [b] This measurement was made at 390 nm.

TABLE V: Effect of Ionic Strength and pH on the Rate of DG Transfer.

	rate (s^{-1}) at pH		
NaCl concn (M)	6.5	7.4	8.5
0.03	4.05	4.15	4.41
0.15	3.87	3.98	3.79
1.5	1.24	1.38	1.44

The effects of pH and ionic strength are shown in Table V. No significant variations in transfer rates were observed at pH 6.5, 7.4, or 8.5. In all cases, the rates were greatly reduced by 1.5 M NaCl.

Discussion

The localization of DG within HDL is supported by the E/M of 0.29, the value found for 3% DG in HDL (Table I). If DG

were distributed uniformly in the bulk phase, the overall concentration would be 1.5 μM with an E/M of 0.002, predicted on the basis of comparison with pyrene in organic solvents. We conclude that the microscopic concentration of DG in HDL is orders of magnitude greater than the macroscopic concentration of DG.

The possibility that DG added to HDL formed separate aggregates distinct from HDL can also be excluded. At 1 mg mL^{-1} HDL containing 3% DG with an E/M of 0.29 (Table I), the bulk concentration of DG, if uniformly distributed, would be 150 μM. The value for E/M actually measured for a DG dispersion at this concentration was approximately 20.

It is remarkable that the rates of DG transfer between HDL were independent of the HDL concentration over a 100-fold range. These results imply that collision of labeled and unlabeled HDL is not the rate-limiting step. A mechanism, consistent with our data, involves dissociation of DG from HDL as the rate-limiting step, followed by rapid diffusion through the aqueous compartment and uptake by the acceptor, unlabeled HDL. If this mechanism is operative, then the transfer of DG from labeled HDL to the aqueous compartment should be similar to that for the transfer of DG from a labeled to an unlabeled HDL. Comparison of the rate constants given in Table III shows good agreement for the transfer rate of these two processes.

In our earlier work we mixed HDL with a solution saturated with pyrene and had estimated that, as the lower limit, the direct uptake of pyrene by HDL was faster than 2 ms. In the present study the low solubility of DG in the aqueous compartment precluded this experiment. However, by analogy and a similarity of all other aspects of this study to that of our earlier study with pyrene and HDL, we expect the transfer of DG, like that of pyrene from the aqueous compartment of an unlabeled HDL particle, to be very rapid.

An alternative mechanism is the rapid formation of a complex between two HDL particles followed by rate-determining lateral diffusion of the fluorophore through the continuous lipid region:

$$\mathrm{HDL^{2*} + HDL} \underset{k_c}{\overset{k_a}{\rightleftharpoons}} \mathrm{HDL^{2*} - HDL} \xrightarrow{k_{meas}} \mathrm{HDL^{*} - HDL^{*}} \underset{k_a}{\overset{k_d}{\rightleftharpoons}} \mathrm{2HDL^{*}}$$

where an asterisk represents fluorescent label. However, in order for the reaction not to be second order with respect to HDL, k_a, the association constant, would have to be much greater than the measured rate constant, k_{meas}. For measurable equilibration to occur, k_{meas} should be comparable or greater than the dissociation constant k_d. Consequently k_a would be very much greater than k_d and the equilibrium constant for formation of HDL dimers would also be much greater than 1. The conclusion is either that HDL forms dimers of approximately 400 000 daltons or that HDL as isolated is a dimer of two 100 000 dalton entities. There is no experimental evidence for either of these possibilities. In addition, a dilution experiment will cause dissociation, where the rate constant, k_d, for the hypothetical complex would be different from k_{meas}; if this were so, it is not clear why there should be fluorescence changes. For these reasons we reject this mechanism.

If the dissociation of DG is rate limiting, the activation energy for this process may be interpreted most simply as equivalent to the free energy of transfer of DG from HDL to the aqueous compartment (Davies & Rideal, 1963).

$$E_a = \mu^\circ_{HDL} - \mu^\circ_{aq}$$

This free energy can also be related to the aqueous concentration of DG, $[DG]_{aq}$, by the following equation (Hall & Pethica, 1967).

$$\mu^\circ_{HDL} - \mu^\circ_{aq} = RT \ln [DG]_{aq}$$

From the value of E_a below 37 °C (Figure 4), the aqueous concentration of DG is calculated to be 2×10^{-9} M. While this value does compare reasonably well with the solubility derived from dilution experiments (2×10^{-8} M), it suggests that the above treatment is too simple. In particular there may be a positive contribution to the activation energy from rearrangement of surface components on loss of DG, which would depress the apparent aqueous concentration of DG.

If the aqueous concentration of DG is defined by the equilibrium between the association and dissociation rates and if the association rate is constant, e.g., diffusion controlled, then the dissociation rate reflects primarily the solubility. Thus increased solubility would give an increased rate. The solubility of a nonelectrolyte depends inversely on the medium polarity. The rate increases observed in 5% Me_2SO and the rate decreases found in the presence of high salt concentrations can thus be interpreted in terms of changes in the solvent polarity. There also may be some alteration in the lipoprotein structure associated with these solvents, compared with the standard buffer usually employed. It is not known what effects these changes may have on the dissociation rate.

We noted that only about 80% of the initial fluorescence changes could be accounted for by a single exponential decay. The reasons for this are not known with certainty, but may be related to the heterogeneous nature of HDL, with subfractions of HDL having different rates of transfer. HDL_3 is a major subfraction of HDL; transfer between HDL_3 was 40% slower than between HDL. On the basis of this evidence, it is reasonable to interpret the transfer as a first-order process with deviations from first-order behavior being due to the heterogeneity of the HDL.

The following scheme proposed for transfer of a probe molecule, DG, is:

$$[DG_1]_{LP_d} \underset{k_a}{\overset{k_d}{\rightleftharpoons}} [DG_2]_{aq} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [DG_3]_{LP_a} \qquad (1)$$

where $[DG_1]_{LP_d}$ is DG concentration at time, t, contained in the donor lipoprotein population, LP_d; $[DG_2]_{aq}$ is DG concentration at time, t, in the aqueous compartment; $[DG_3]_{LP_a}$ is DG concentration at time, t, contained in the acceptor lipoprotein population, LP_a; k_d is the first-order rate constant for dissociation; k_a is the pseudo-first-order rate constant for association.[2]

General solutions derived from this type of kinetic scheme are known (Frost & Pearson, 1961). The explicit solution for this system is the following:

$$[DG_1] = [DG_1]^\circ \left[\frac{k_d + fk_a}{f(k_d + 2k_a)} + \tfrac{1}{2}e^{-k_d t} + \frac{k_d(f-2)}{2f(k_d + 2k_a)} e^{-(k_d+2k_a)t} \right]$$

$$[DG_2] = [DG_1]^\circ \left[\frac{k_d + fk_a}{f(k_d + 2k_a)} - \tfrac{1}{2}e^{-k_d t} + \frac{k_d(f-2)}{2f(k_d + 2k_a)} e^{-(k_d+2k_a)t} \right]$$

[2] Association requires the collision of a probe with a lipoprotein particle and is a second order reaction. However, since the lipoprotein is normally present in excess, the pseudo-first-order rate constant, k_a, is used.

where $[DG_1]^\circ$ is the initial DG concentration in the donor lipoprotein and f is a mixing factor.[3] From the partition coefficient, k_a must be very much greater than k_d. A comparison of amplitudes of the two exponentials shows that the second one is negligible and, when $f = 2$ (mixing equal volumes of solution), the second exponential vanishes. The observed rate corresponds to that of dissociation.

The present studies show that the kinetics of the transfer of DG are limited by its solubility in the aqueous phase. The relatively short half-time for transfer of DG compared with the lifetime of the lipoproteins in plasma suggests that the distribution of this class of neutral lipid between lipoproteins is thermodynamically controlled. Future studies will be concerned with the kinetics and distribution of other lipids among the plasma lipoproteins in order to understand the importance of exchange and transfer as factors which affect lipoprotein structure and metabolism.

Acknowledgments

The authors gratefully acknowledge stimulating discussions with Drs. Yin J. Kao, Kirk C. Aune, Joel D. Morrisett, Henry J. Pownall, and John S. Olson. We are especially indebted to Drs. Dominic M. Desiderio and Fred R. Seymour for the mass spectra.

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[3] The mixing factor, f, is 2 if the initial solution is diluted 1:1 and 15, if the dilution is 1:15.