Transport of heptafluorostearate across model membranes. Membrane transport of long-chain fatty acid anions I[1]

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Abstract Heptafluorostearic acid, an isogeometric derivative of stearic acid, has a pK_a value of about 0.5. To evaluate the suitability of heptafluorostearate as model compound for anions of long-chain fatty acids in membrane transport, monolayer and liposome studies were performed with lipid mixtures containing phospholipids–cholesterol–heptafluorostearate or stearate (100:40:20 molar ratios). Transfer of heptafluorostearate and stearate from liposomes to bovine serum albumin (BSA) was followed by measuring the intrinsic fluorescence of BSA. The percentage of heptafluorostearate, equivalent to the amount placed in their outer monolayer, transferred from liposomes (120–130 nm diameter) to BSA was 55.7 ± 3.7% within 10 min at 25°C and 55 ± 2% within 5 min at 37°C. Slow transfer of 22.7 ± 2.5% of heptafluorostearate at 25°C followed with a half-life of 2.3 ± 0.4 h and of 20 ± 4% at 37°C with a half-life of 0.9 ± 0.1 h until the final equilibrium distributions between BSA and liposomes were reached, 79 ± 6% to 21 ± 5% at 25°C and 75 ± 5% to 25 ± 4% at 37°C. The pseudounimolecular rate constants for flip-flop of heptafluorostearate equal $k_{FF,25} = 0.24 \pm 0.05\ h^{-1}$ and $k_{FF,37} = 0.6 \pm 0.1\ h^{-1}$, respectively. By comparison, transfer of stearate required only 3 min to reach equilibrium distribution. The difference between heptafluorostearate and stearate may be explained by a rapid flip-flop movement of the un-ionized fatty acids which exist in different concentrations in accordance with their pK_a values. Half-life of flip-flop of heptafluorostearate makes it suitable to study mediated membrane transport of long-chain fatty acid anions.—Schmider, W., A. Fahr, H. E. Blum, and G. Kurz. **Transport of heptafluorostearate across model membranes. Membrane transport of long-chain fatty acid anions. I.** *J. Lipid Res.* **2000.** 41: **775–787.**

Supplementary key words flip-flop mechanism • intrinsic fluorescence • liposome • long-chain fatty acid • monolayer • transbilayer movement

The physicochemical properties of long-chain fatty acids, their pK_a values, their extreme low solubilities in aqueous solutions, their tendency to form micelles, and their fast intracellular metabolism, make the studies of their transport across biological membranes very difficult. Due to the pK_a values of long-chain fatty acids, under physiological conditions small fractions of the compounds are present uncharged and can permeate lipid bilayers by simple diffusion (1–6). The study of mediated transport of anions of long-chain fatty acids, therefore, is relatively complicated. In addition, the extremely low solubility of long-chain fatty acids and their salts in aqueous solutions requires binding proteins like albumin, adding to the complexity (7, 8).

In order to bypass the difficulties arising from the susceptibility of long-chain fatty acid anions to protonation as well as metabolism, an isogeometric derivative of stearate, heptafluorostearate, has been synthesized (9). This derivative has a pK_a value of the corresponding acid of about 0.5, and is taken up into isolated hepatocytes by mediated transport (10). The chemically convincing proof of the existence of transporters of long-chain fatty acid anions requires laborious photoaffinity labeling studies. Thus, the availability of an analogue of long-chain fatty acids, which allows a simple and direct proof of mediated uptake in any given cell type, is urgently needed.

A comparison of the reversible transfer of heptafluorostearate and stearate, respectively, between liposomes and albumin should give insight into the mechanism of transport across lipid bilayers. Based on the assumption that long-chain fatty acid anions permeate lipid bilayers in

Abbreviations: BSA, bovine serum albumin; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPG, 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; Heptafluorostearate, 2,2,3,3,18,18,18-heptafluorooctadecanoate; [^{3}H]-heptafluorostearate, 2,2,3,3,18,18,18-heptafluoro[G-^{3}H]octadecanoate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol.

[1] In the present communication, the term fatty acid anion does not denote any state of ionization and comprises both the anion and its corresponding acid. The term fatty acid will be used only when the protonated uncharged form is taken into consideration.

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their protonated form by a flip-flop mechanism (1, 2, 4, 5, 11), stearate and its analogue are expected to behave differently. In view of the extremely small protonated fraction of heptafluorostearate, simple diffusion is of minor importance in membrane transport under physiological conditions, making the isogeometric analogue of stearate ideal for the detection and the analysis of mediated transport of long-chain fatty acid anions through biological membranes.

MATERIALS AND METHODS

Materials

Silica gel plates (silica gel 60, 10 × 20 cm) for thin-layer chromatography were obtained from E. Merck (Darmstadt, Germany). Stearic acid was from Aldrich GmbH & Co. KG (Steinheim, Germany). BSA (bovine serum albumin, fraction V, essentially fatty acid-free) and DSPG were from Sigma Chemie GmbH (Deisenhofen, Germany). Cholesterol, POPC, POPG, and DSPC were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Sodium heptafluorostearate and sodium [^{3}H]heptafluorostearate (2.63 TBq/mmol) were synthesized and characterized as described (9). [1-^{14}C]stearic acid (2.15 GBq/mmol) was purchased from Amersham Buchler (Braunschweig, Germany), and cholesteryl [1-^{14}C]oleate (2.04 GBq/mmol) was from Biotrend Chemikalien GmbH (Köln, Germany). The purity of all compounds was routinely controlled by thin-layer chromatography as described (12). All other chemicals were of the highest quality commercially available.

Buffer

For preparation of buffer, water from a Milli-Q Plus water purification system (Millipore GmbH, Eschborn, Germany) with a resistivity of 18.2 MΩ·cm was used. If not stated otherwise, all experiments were performed in 50 mm sodium phosphate buffer, pH 7.4, containing 150 mm sodium chloride and 0.02% (w/v) sodium azide.

Determination of protein concentration and radioactivity

Protein concentrations were determined spectrophotometrically with a UV/VIS-spectrophotometer Uvikon 940 (Kontron Technik GmbH, Eching, Germany), based on the extinction coefficient of $\varepsilon_{280} = 0.044\ \mu m^{-1} \cdot cm^{-1}$ at 280 nm for BSA (13).

Determination of radioactivity was carried out with a Tri-Carb liquid scintillation spectrometer 3375 (Canberra Packard International S. A., Zürich, Switzerland) or with a liquid scintillation counter Wallac 1411 (Berthold, Wildbad, Germany) using the cocktails Lumagel® or Ultima Gold® (Canberra Packard International S. A.).

Quantification of fatty acid anions by BSA binding

Total concentrations of fatty acid anions were determined making use of the decrease of intrinsic fluorescence of BSA upon binding of the ligands (14–16). Measurements of the intrinsic fluorescence of BSA were carried out using a Luminescence Spectrometer LS 50 B (Perkin-Elmer, Überlingen, Germany) and 1-cm fluorescence cuvettes containing a stirring bar. The fluorescence intensities were measured at 348 nm with a slit width of 5 nm using excitation at 300 nm and a slit width of 5 nm. In all experiments, 2.8 ml of a 3 μm solution of BSA in buffer were stirred at a constant rate of 750 rpm and were kept at the desired temperature of 25° or 37°C with a maximal deviation of ±0.5°C.

In order to determine the quenching of the intrinsic fluorescence of BSA upon binding of the corresponding fatty acid anion, the fluorescence intensity of BSA was first measured in the absence of the ligand, after incubation for 10 min at the chosen temperature. Then, an aliquot of an ethanolic stock solution of the corresponding compound was added. In order to allow a simple and independent determination of total concentration, the stock solution had a defined specific radioactivity. The resulting fluorescence intensity was measured after an incubation period of 5 min. Total concentration of the respective compound in the solution was determined from the radioactivity in an aliquot of the test mixture each time after measuring the fluorescence intensity. The addition of the ethanolic stock solution was repeated several times, never exceeding a maximal final ethanol concentration of 0.8% (v/v) in the BSA solution. In control experiments, this concentration of ethanol was shown to have no detectable effect on the intrinsic fluorescence of BSA and its interaction with fatty acid anions.

Under the experimental conditions used, the dependency of the intrinsic fluorescence of BSA from the concentration of total fatty acid anions in the solution can be best described by the empirical equation 1:

$$f_{(c)} = a_1 + a_2 \cdot e^{-a_3(a_4 + c)^2}. \qquad \textit{Eq. 1}$$

The fluorescence intensities in the presence of ligands are related to the fluorescence intensities of BSA in the absence of ligands and are expressed in percent of initial fluorescence as $f_{(c)}$. Symbol c represents the total concentration of long-chain fatty acid anions; the parameters a_i have no physical meaning and are only necessary for the mathematical description of the data. Under the experimental conditions used, equation 1 is applicable for $c \leqslant 12\ \mu m$. For fast numerical analysis of the many data equation 1 was transformed into its inverse form, shown by equation 2:

$$c = \sqrt{-\frac{\ln\left(\frac{f_{(c)} - a_1}{a_2}\right)}{a_3}} - a_4. \qquad \textit{Eq. 2}$$

To simplify the graphic representation, percent intrinsic fluorescence in equation 2 was substituted by percent quenching, $q_{(c)}$, defined as $q_{(c)} = 100 - f_{(c)}$.

Monolayer experiments

Determination of molecular areas was made either in a circular trough (type RMC2-T, Mayer Feinmechanik, Göttingen, Germany) or in a rectangular one (type Biotrough, Riegler und Kirstein, Wiesbaden, Germany) with the aid of a computer-controlled film balance (17, 18). The subphases in the circular and in the rectangular trough consisted of 40 ml and 35 ml of buffer, respectively, and were kept at a temperature of 25 ± 0.5°C. Molecular areas of heptafluorostearate and stearate were compared in monolayers of the pure compounds and in those of an appropriate lipid mixture. The fatty acid anions were dissolved in n-hexane–ethanol 9:1 (v/v) at concentrations of 1.75 or 3.3 mm. Lipid mixtures were prepared containing POPC–POPG–DSPC–cholesterol (90:5:5:40 molar ratios) and POPC–POPG–DSPC–cholesterol–heptafluorostearate or stearate (90:5:5:40:20 molar ratios) in n-hexane–ethanol 9:1 (v/v) to a final concentration of cholesterol of 0.6 mm. After spreading an appropriate volume of the respective solutions on the maximal surface areas of 72 cm^2 in the circular trough and 56 cm^2 in the rectangular trough, the films were kept at the expanded state for 15 min. Subsequently, compression was performed at a speed of 10 or 20 Å^2/(molecule·min).

Preparation and characterization of liposomes

Liposomes were prepared by the extrusion method as described (1, 19, 20), using an extrusion device LiposoFast™ (Milsch-Equipment, Laudenbach, Germany) equipped with

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polycarbonate filters of pore diameters of either 200 or 100 nm (Milsch-Equipment). Routinely, the samples were first extruded 21 times through one filter with a pore diameter of 200 nm and subsequently 21 times through two filters in tandem having a pore diameter of 100 nm. Loaded liposomes used for the study of transfer of fatty acid anions from liposomes to BSA were composed of POPC–POPG–DSPC–cholesterol–heptafluorostearate or stearate (90:5:5:40:20 molar ratios) or of POPC–DSPG–cholesterol–heptafluorostearate or stearate (95:5:40:20 molar ratios). The lipid composition of the liposomes used for the study of transfer of fatty acid anions from BSA to liposomes was POPC–POPG–DSPC–cholesterol (90:5:5:40 molar ratios) or POPC–DSPG–cholesterol (95:5:40 molar ratios). The amount of lipids used for preparation of liposomes varied usually between 2 and 10 mg of cholesterol equivalents per ml of final suspension.

The lipid concentration in the liposomal suspensions was controlled enzymatically by determination of cholesterol (21) using the CHOD-CAT method (Roche Diagnostics GmbH, Mannheim, Germany) and choline-containing phospholipids (22) using the Phospholipids B test system (WAKO Chemicals GmbH, Neuss, Germany). The concentration of fatty acid anions was determined in all liposomal preparations by liquid scintillation counting making use of fatty acid anions of a defined specific radioactivity. The size distribution of the liposomes was measured by dynamic laser light scattering with a ZetaSizer III (Malvern, England).

The amount of lipid present in the outer monolayer of the liposomes was determined by ^{31}P- or ^{1}H-NMR spectroscopy in the absence and the presence of 5 or 1 mm Mn^{2+}, respectively, principally as described (19, 23) after removing of untrapped phosphate buffer by gel filtration or ultracentrifugation.

Transfer experiments

Transfer of fatty acid anions between liposomes and BSA was followed in 1-cm cuvettes by the change of the intrinsic fluorescence of BSA upon interaction with the ligands, using excitation at 300 nm with a slit width of 5 nm and emission at 348 nm with a slit width of 5 nm. After adjustment of the setups to the temperature of the experiments for 10 min, fluorescence intensity was continuously recorded for 400 s or sometimes for 600 s in 1-s intervals and then for 8 h in 90-s intervals. For control, measurements circumventing photodecomposition of BSA were performed by opening the shutter at discrete times only. After following the initial fluorescence for 50 s, transfer was started by addition of liposomes. Complete mixing was shown to be reached within 5–7 s after addition of the liposomes in $<$1 s. For the study of transfer of fatty acid anions from liposomes to BSA, usually 2.7 ml of a 3.11 μm BSA solution, stirred with 750 rpm, was mixed with 0.1 ml of loaded liposomes corresponding to a cholesterol concentration of 0.5 mm. Taking into account the dilution factor of 0.964, all transfer experiments were performed with 3 μm BSA, liposomes corresponding to a cholesterol concentration of 18 μm, and a total concentration of fatty acid anions of 9 μm.

To determine the influence of the addition of liposomes to the BSA solution on the fluorescence, in control experiments 2.7 ml of a 3.11 μm BSA solution was mixed with 0.1 ml liposomes containing no fatty acid anions. To take into account the decrease of the fluorescence by photodecomposition, time dependency of the fluorescence of a 3 μm BSA solution containing either heptafluorostearate or stearate in different concentrations varying between 3 and 9 μm was determined.

Data analysis

Parameters of functions describing calibration plots and fatty acid anion transfer were calculated by the nonlinear least-squares regression analysis program Slide Write Plus 2.0 (Advanced Graphics Software, Carlsbad, CA).

For quantitative analysis of transfer, the measured fluorescence intensities were divided after normalization by the corresponding fluorescence intensities of the controls, thereby eliminating effects of dilution and photodecomposition. Multiplication of these quotients, which correspond to transfer only, by the percentage of initial BSA fluorescence, related to the solution of BSA in the absence of fatty acid anions, resulted in the percent of initial fluorescence $f_{(c)}$ at time t. Substitution of $f_{(c)}$ into equation 2 resulted in the extra-liposomal concentration c of the respective fatty acid anion at time t. Concentrations of fatty acid salt anions were calculated from the measured fluorescence intensities with the aid of the table calculation program Lotus 1-2-3 for Windows 1.1 (Lotus Development Corporation, Staines, Middlesex, England). For evaluation data, points from 5 s on were taken into consideration.

The course of concentration of a long-chain fatty acid anion moving from a donor to an acceptor may be described by equation 3 for the donor compartment:

$$c_{D(t)} = \sum_{i=1}^{n} A_{D,i} \cdot e^{-k_i \cdot t} + C_{D,\infty} \qquad \textit{Eq. 3}$$

and by equation 4 for the acceptor compartment:

$$c_{A(t)} = \sum_{i=1}^{n} A_{A,i} \cdot (1 - e^{-k_i \cdot t}), \qquad \textit{Eq. 4}$$

where $A_{D,i}$ represents the amplitudes of the respective exponential functions for the donor compartment and $A_{A,i}$ those for the acceptor compartment; k_i represents the corresponding rate constants; and n is the number of exponential functions. The resulting parameters are reported as means ± SD. Statistical differences were determined by Student's *t*-test. For comparison of the selected models for mathematical description of transfer curves, the runs test (24) and the Akaike criterion (25) were applied.

Programs for conversion of measured fluorescence intensities into concentrations and for calculation of data points for estimation of rate constants are available from the authors upon request.

RESULTS

Comparison of heptafluorostearate and stearate in lipid monolayers

In order to compare heptafluorostearate and stearate in lipid monolayers and to estimate the electronic and steric effects of the introduction of 7 fluorine atoms, the surface pressure–area isotherms of the pure compounds at the air/buffer interface were recorded (**Fig. 1**). The surface pressure–area isotherm of heptafluorostearate differs at pH 7.4 of the subphase clearly from that of stearate, but exhibits a similar shape as stearate at pH = 13 at pressures $\leq$20 mN/m. The difference in the surface pressure–area isotherm of heptafluorostearate at pH 7.4 and stearate at pH 13 at pressures $>$20 mN/m probably reflects differences in the interaction of the counter-ions of the subphase with the carboxylate groups of the fatty acid anions. The molecular area of heptafluorostearate was estimated on the basis of the equations of state for ionized monolayers derived by Davies (26) and by Fowkes (27). Using the experimental data for surface pressures $\leq$20 mN/m, the molecular area of heptafluorostearate at pH 7.4 was calculated with both equations to be 30 ± 2 Å^2/molecule and 29 ± 2 Å^2/molecule, respectively. Under the experimental conditions used, the molecular area

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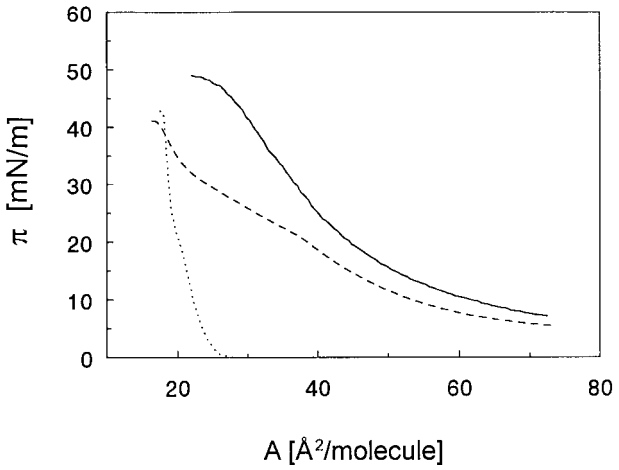

Fig. 1. Surface pressure–area isotherm of heptafluorostearate and stearate at 25°C. The subphase consisted of 40 ml of 50 mm sodium phosphate buffer, pH 7.4, containing 150 mm sodium chloride and 0.02% (w/v) sodium azide or of 0.1 m NaOH, pH 13, containing 180 mm NaCl and 0.02% (w/v) sodium azide. (—) Heptafluorostearate at pH 7.4, (· · · ·) stearate at pH 7.4, (- - - -) stearate at pH 13.

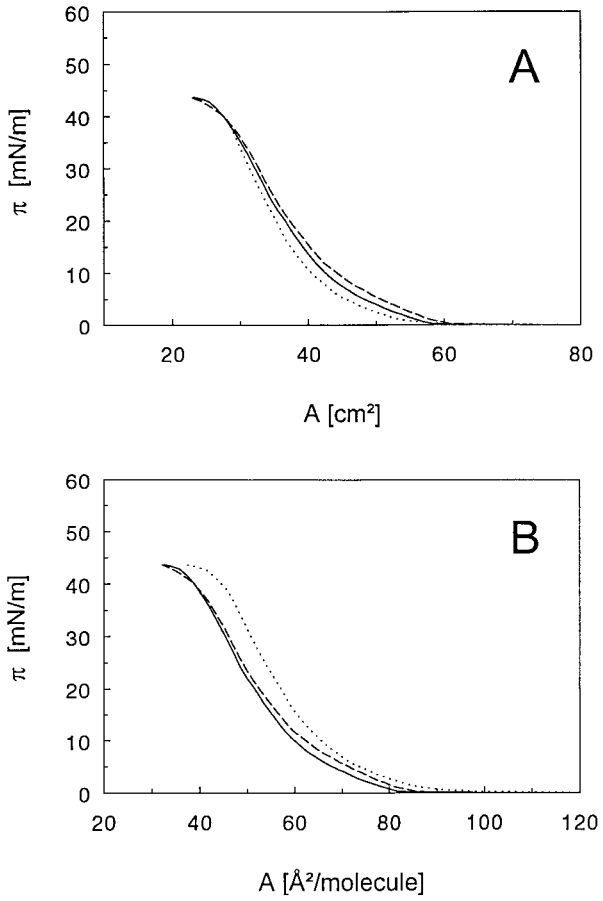

Fig. 2. Surface pressure–area isotherms of different lipid mixtures at 25°C. The subphase consisted of 40 ml of 50 mm sodium phosphate buffer, pH 7.4, containing 150 mm sodium chloride and 0.02% (w/v) sodium azide. (· · · ·) Lipid mixture containing POPC–POPG–DSPC–cholesterol (90:5:5:40 molar ratios); (—) lipid mixture containing additionally 12.5 mol% heptafluorostearate; (- - - -) lipid mixture containing additionally 12.5 mol% stearate. The surface pressure is plotted versus the total area of the film (A) and versus the mean molecular area per molecule (B).

of stearate was determined by simple graphic analysis (28) to be 20 ± 1 Å²/molecule at pH 7.4 and, dependent on the equation used, 25–33 Å²/molecule at pH 13, in agreement with published data (28–31).

In order to compare the influence of heptafluorostearate or stearate incorporated into a lipid mixture, the surface pressure–area isotherms of the same lipid mixture as used for the preparation of liposomes were recorded in the absence and the presence of 12.5 mol% of both compounds (**Fig. 2**). As compared with the lipid mixture containing no fatty acid anions, the typical surface pressure–area isotherms of the mixtures of POPC–POPG–DSPC–cholesterol–fatty acid anion (90:5:5:40:20 molar ratios) are slightly but not significantly shifted to increased areas (Fig. 2A). The presence of 12.5 mol% of heptafluorostearate or stearate has only a relatively small effect on the molecular area in the lipid mixture and differences between the two fatty acid anions are not detectable within the experimental error. The analogy of heptafluorostearate and stearate becomes more obvious by plotting the pressure versus the mean molecular area (Fig. 2B), demonstrating a significant decrease of the mean molecular area in presence of both fatty acid anions.

Determination of heptafluorostearate and stearate by fluorescence of BSA

In order to correlate the intrinsic fluorescence of BSA with the total concentration of heptafluorostearate or stearate under the conditions used for following the transfer of the fatty acid anions between liposomes and BSA, fluorescence emission at 348 nm was measured, using the excitation wavelength of 300 nm. The dependency of the intrinsic fluorescence of 3 μm BSA from total concentrations of heptafluorostearate (**Fig. 3A**) or stearate (Fig. 3B) was determined at 25 and 37°C, respectively. Total concentrations of the fatty acid anions varied between 0.3 and 24 μm corresponding to molar ratios of fatty acid anions to BSA from 0.1 to 8.

Up to concentrations of the fatty acid anions of 12 μm, all experimentally obtained quenching curves followed the empirical equation 1 for a Gaussian function with the parameters listed in **Table 1**. In its inverse form (equation 2) this equation allows the simple numerical analysis of many data. The excellent fit of equation 2 for the indicated concentration range in the c-versus-$q_{(c)}$ diagram obtained with heptafluorostearate (**Fig. 4A**) and stearate (Fig. 4B) demonstrates its suitability.

Characterization of liposomes

The liposomes used for the study of transfer of fatty acid anions from liposomes to BSA contained phospholipids–cholesterol–heptafluorostearate or stearate (100:40:20 molar ratios), the proportion of fatty acid anions being equivalent to 12.5 mol%. As demonstrated by dynamic laser

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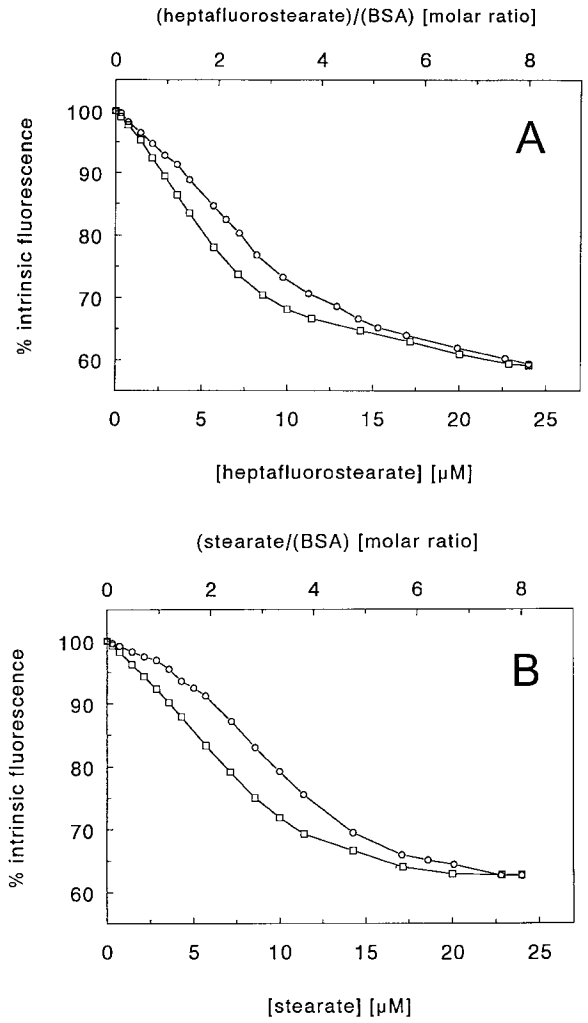

Fig. 3. Dependency of the intrinsic fluorescence of 3 μm BSA in 50 mm sodium phosphate buffer, pH 7.4, containing 150 mm sodium chloride and 0.02% (w/v) sodium azide, from total concentration of heptafluorostearate (A) or stearate (B). Fluorescence emission was measured at 348 nm with a slit width of 5 nm using excitation at 300 nm and a slit width of 5 nm; the temperature was 25 (○) and 37°C (□). Total concentrations of fatty acid anions are plotted on the abscissa on the bottom, the corresponding molar ratios of total fatty acid anions to BSA are plotted on the abscissa on the top.

light scattering, liposomes composed of POPC–POPG–DSPC–cholesterol–heptafluorostearate or stearate (90:5:5:40:20 molar ratios) or POPC–DSPG–cholesterol–heptafluorostearate or stearate (95:5:40:20 molar ratios) had a mean diameter of 120–130 nm. All liposomal preparations showed polydispersities <0.05.

In order to estimate the amount of total lipid in the outer liposomal monolayer, the portion of phospholipids facing the extra-liposomal solution was determined from the comparison of the ^{31}P- or ^{1}H-NMR signals in the absence and the presence of Mn^{2+}. The ^{31}P-NMR spectra of the resulting liposomal suspensions showed one signal originating from the phosphorus atoms in the phospholipids and the other from phosphate ions entrapped within the liposomes as well as from those not completely separated by gel filtration or centrifugation. Out of the ^{1}H-NMR signals, that of the methyl groups of the choline moiety in POPC was used for interpretation. After addition of Mn^{2+}, the NMR signals of the phospholipids of unloaded liposomes as well as of fatty acid anion-loaded liposomes were lowered by 50 ± 3%, indicating that this portion of total lipid is located in the outer liposomal monolayer. On the basis of a bilayer thickness of 4.5 nm (32), the amount of lipid located in the inner liposomal monolayer of liposomes with a mean diameter of 120–130 nm is about 43% of total lipid. That means that about 93% of total lipid are actually present in the liposomal membrane and only about 7% of the membranes are not directly accessible due to the existence of oligolamellar structures.

Transfer of heptafluorostearate and stearate from liposomes to BSA

In order to determine the time required to reach the equilibrium distribution of heptafluorostearate and stearate between liposomes and BSA, transfer was followed by the change in intrinsic fluorescence of BSA. Transfer of heptafluorostearate from loaded liposomes to BSA at 25°C (**Fig. 5**), started by the addition of the loaded liposomes, caused a fast decline of intrinsic fluorescence within the first minutes, followed by a slow decrease over several hours until equilibrium was nearly reached. In all experiments the final equilibrium was determined for control after 24 h (Fig. 5B).

Continuous determination of the intrinsic fluorescence of BSA requires long exposure times to light. In order to determine the photodecomposition of BSA under the experimental conditions of transfer, the intrinsic fluorescence of the protein was followed in the absence and in the presence of the other components (**Fig. 6**). BSA showed a small time-dependent decrease in intrinsic fluorescence of 0.55 ± 0.05%/h of initial fluorescence, which is not affected by the presence of empty liposomes. The

TABLE 1. Parameters of c-versus-$q_{(c)}$ diagram of the quenching of intrinsic fluorescence of BSA in the presence of heptafluorostearate (A) or stearate (B) at 25 and 37°C

(A) Parameters of c-versus-$q_{(c)}$ Diagrams of Heptafluorosterate					(B) Parameters of c-versus-$q_{(c)}$ Diagrams of Stearate				
Temperature	a_1	a_2	a_3	a_4	Temperature	a_1	a_2	a_3	a_4
°C	%	%	μM^{-2}	μM	°C	%	%	μM^{-2}	μM
25	63.3	39.3	0.008	2.90	25	58.0	41.8	0.006	0.83
37	64.4	40.0	0.015	2.80	37	64.0	38.6	0.010	2.83

The data from Figs. 4A and B were fitted with equation 2. Fit quality for all sets of parameters was assessed by correlation coefficients > 0.997.

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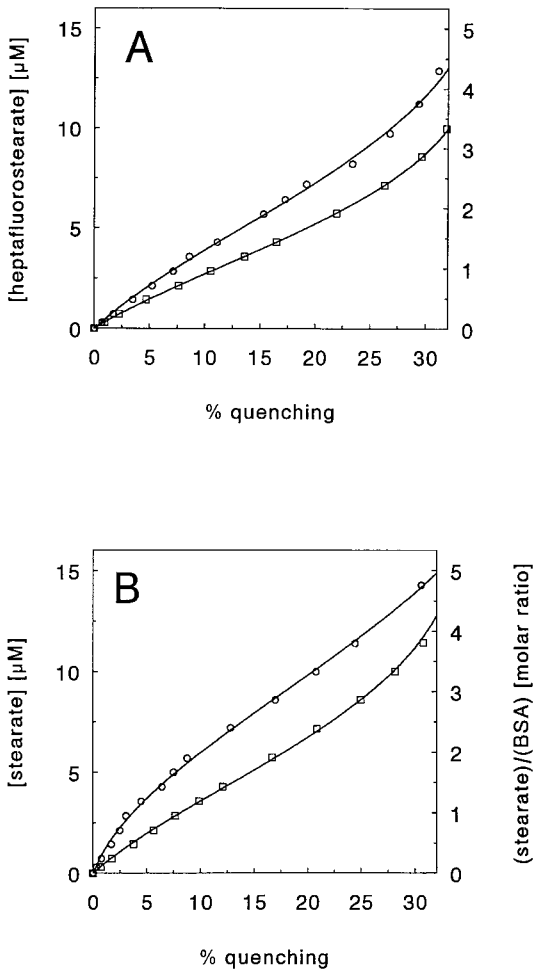

Fig. 4. c-versus-$q_{(c)}$ diagram of total concentrations of total fatty acid anions (left ordinate) and molar ratios of total fatty acid anions to BSA (right ordinate) for heptafluorostearate (A) and stearate (B) at 25 (○) and 37°C (□) as function of the determined quenching; data converted from Fig. 3. The curves are based on equation 2 and the corresponding parameters from Table 1A and B.

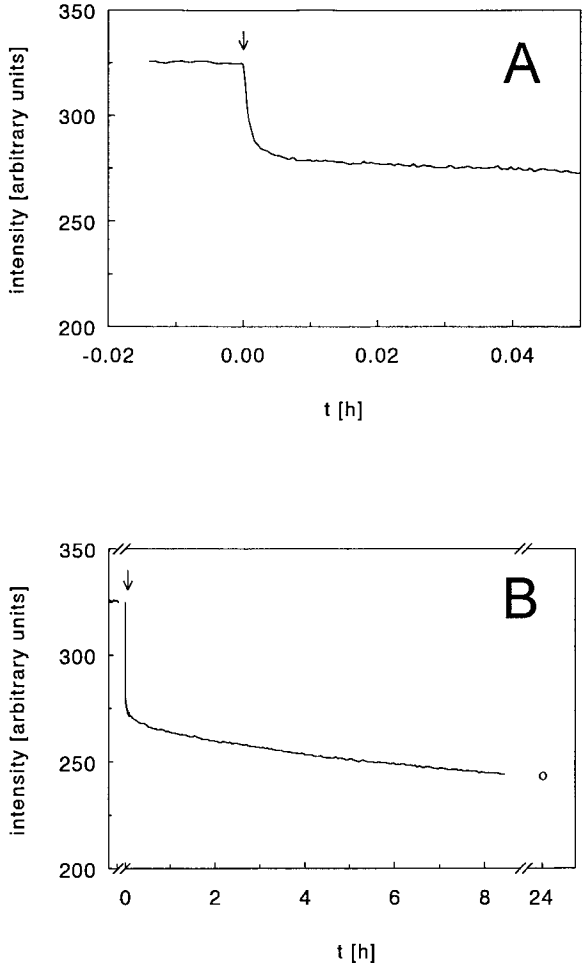

Fig. 5. Time-dependent change in intrinsic fluorescence of BSA during transfer of heptafluorostearate from liposomes to BSA at 25°C. (A) First 3 min and (B) complete time course. After following the intrinsic fluorescence of BSA for 50 s, transfer was started by addition of 0.1 ml of liposomes loaded with 12.5 mol% heptafluorostearate, as indicated by the arrow. The solution contained finally 9 μm heptafluorostearate, 3 μm BSA, and liposomes corresponding to a cholesterol concentration of 18 μm. Continuous measuring was stopped after 8.5 h, and the test solution was kept in the dark at 25°C until recording the fluorescence intensity 24 h after start of transfer; (○) indicates the measured fluorescence intensity after 24 h.

presence of 0.35% (v/v) ethanol, required to dissolve heptafluorosterate for incubation with empty liposomes, has an effect on neither the intrinsic fluorescence of BSA nor on the stability of the liposomes. The presence of heptafluorostearate became not only apparent by a clear quenching of the intrinsic fluorescence but also by a decrease of fluorescence by photodecomposition of 0.38 ± 0.04%/h of initial fluorescence, slightly but significantly ($P < 0.01$) different from that of the solution of pure BSA.

Immediately after completion of mixing, photodecomposition was still insignificant. To evaluate the influence of scattering on fluorescence intensity of BSA, the relative decrease of fluorescence intensities was compared 1 min after addition of either empty liposomes or buffer to pure BSA or of buffer to BSA preloaded with heptafluorostearate. In all cases the relative decrease in fluorescence intensity was identical within the experimental error, indicating that scattering is of no significant influence.

In order to obtain the change in fluorescence intensity caused exclusively by transfer of heptafluorostearate, the effects resulting from dilution and photodecomposition were automatically taken into account with the aid of a table calculation program. Because photodecomposition of BSA shows a small dependency of ligand concentration, as a good approximation the time dependencies of photodecomposition of BSA taken into account for baseline subtraction were related to fatty acid anion concentrations, reached after one half-life period of the slow transfer process under the respective experimental conditions. The error introduced by ignoring the changing concentrations of the fatty acid anion is less than 1% of the recorded intensities. The changes of fluorescence intensity due to transfer of heptafluorostearate were converted into concentrations with the aid of the calibration plot (Fig.

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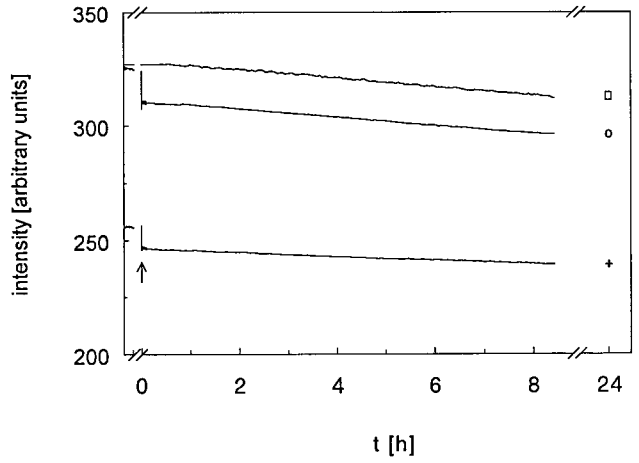

Fig. 6. Time-dependent decrease of the intrinsic fluorescence of BSA due to photodecomposition at 25°C. The fluorescence intensities were measured at 348 nm with a slit width of 5 nm using excitation at 300 nm and a slit width of 5 nm. The symbols indicate the fluorescence intensities measured after 24 h: (□) solution of 3.11 μm BSA in buffer; (○) BSA after addition of liposomes, as indicated by the arrow, corresponding to a final concentrations of cholesterol of 18 μm and of BSA of 3 μm in the presence or absence of 0.35% (v/v) ethanol; (+) solution of heptafluorostearate and BSA, after addition of 0.1 ml buffer, as indicated by the arrow, final concentrations of 7 μm heptafluorostearate and 3 μm BSA were reached.

4A). The resulting course of transfer shows the dependency either of percent of heptafluorostearate incorporated into liposomal membranes or of its extra-liposomal concentration upon transfer time (**Fig. 7**, solid line).

To address the potential problem of continuous determination of fluorescence and correction for photodecomposition, discontinuous control experiments were performed by opening and closing the shutter only at discrete times.

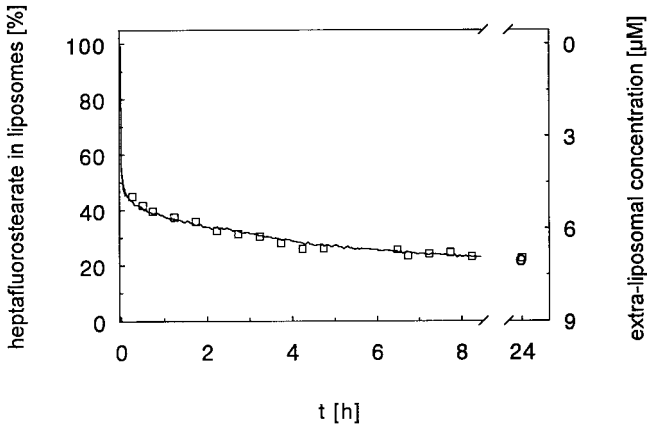

Fig. 7. Time dependency of the transfer of heptafluorostearate from liposomes to BSA at 25°C. The BSA and the heptafluorostearate concentrations were 3 μm and 9 μm, respectively, the concentration of liposomes corresponded to 18 μm cholesterol; (○) indicates the equilibrium value measured after 24 h, as described in the legend of Fig. 5. For control, the change in fluorescence of BSA during transfer of heptafluorostearate was measured discontinuously by opening and closing the shutter at discrete times, and transformed into moles of heptafluorostearate transferred to BSA (□).

Discontinuously determined fluorescence intensities coincide within the experimental error with the respective continuously determined values corrected for photodecomposition and yielded the same course of transfer as obtained by continuous measurements (Fig. 7).

Under the experimental conditions used, in equilibrium, 79 ± 6% of total heptafluorostearate was found to be extra-liposomal and only about 21 ± 5% was incorporated in liposomal membranes. Examination of time course of transfer of heptafluorostearate from loaded liposomes to BSA revealed that the transfer could be freely subdivided into two phases: a fast first phase comprising transfer of 55.7 ± 3.7% and a subsequent slow phase making up 22.7 ± 2.5% of total heptafluorostearate (Fig. 7).

For comparison, transfer of stearate from liposomes to BSA was analyzed by recording the time-dependent change in the intrinsic fluorescence of BSA under conditions identical to those with heptafluorostearate. Transfer of stearate caused a fast decline of intrinsic fluorescence of BSA and reached, in contrast to heptafluorostearate, the final equilibrium within 3 min (**Fig. 8A**). The final fluorescence intensity was again controlled after interruption of exposure to light and proved to be stable for more than 24 h. Due to the short time interval needed to reach the final equilibrium, for transformation of measured fluorescence intensities into the course of transfer, photodecomposition could be neglected and only the effect of dilution had to be taken into account. Transformation of fluorescence intensities using the corresponding calibration plot (Fig. 4) yielded the course of transfer of stearate from liposomes to BSA (Fig. 8B). Transfer of stearate from liposomes to BSA reached the final distribution equilibrium, 85 ± 6% of stearate bound to BSA and 15 ± 4% incorporated into the liposomes, in only one fast phase.

Identification of transversal movement of heptafluorostearate

The identity of the slow phase of transfer of heptafluorostearate (Fig. 7) with its transversal movement, suggested by the percentage transferred, was studied by comparison of short- and long-term incubation of empty liposomes with heptafluorostearate. For these studies the dissolution of heptafluorostearate in an appropriate non-aqueous solvent, prior to its addition to the liposomal suspension, turned out to be imperative to ensure the absolutely necessary fast and complete dissolution of the fatty acid anion. Ethanol in the final concentration of 0.1% (v/v) proved to be adequate and did not affect the qualitative behavior of heptafluorostearate transfer, as judged by studies of transfer of heptafluorostearate from liposomes to BSA.

Incubation of empty liposomes with heptafluorostearate for 24 h led qualitatively to the expected time course during its transfer from the liposomes to BSA (**Fig. 9**). Whereas the amounts of heptafluorostearate transferred during the fast and the slow phase are comparable with those observed in the absence of ethanol (Fig. 7), the half-life of the slow phase was significantly decreased to about 1 h in its presence.

In contrast, transfer of heptafluorostearate from lipo-

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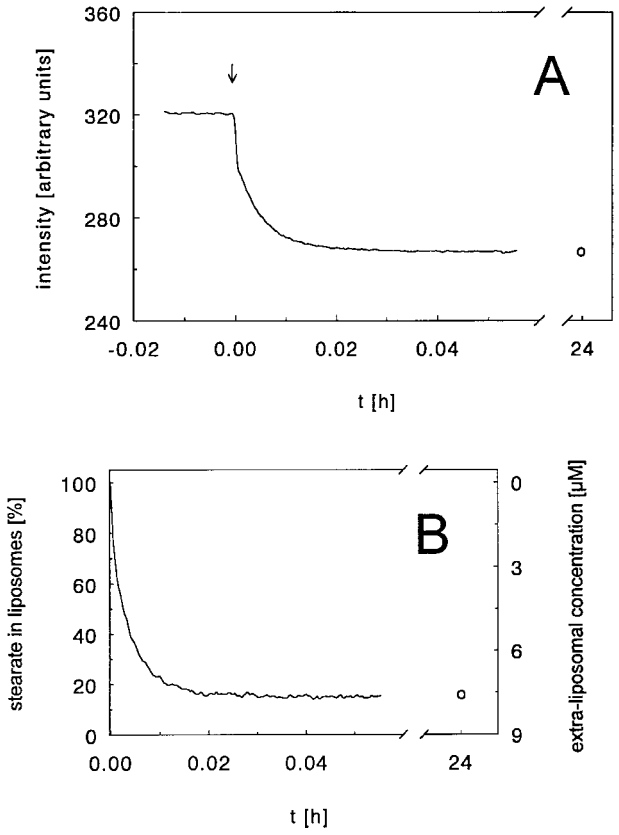

Fig. 8. Transfer of stearate from liposomes to BSA at 25°C. (A) Time-dependent change in intrinsic fluorescence of BSA during transfer. After following the intrinsic fluorescence of BSA for 50 s, transfer was started by the addition of liposomes, as indicated by the arrow, resulting in a solution containing 9 μm stearate, 3 μm BSA, and liposomes corresponding to a cholesterol concentration of 18 μm. Continuous measuring was stopped after 200 s, and the test solution was kept in the dark at 25°C until recording the fluorescence intensity 24 h after start of transfer. Conditions for transfer were the same as described in the legend of Fig. 5; (○) indicates the fluorescence intensity measured after 24 h. (B) Time dependency of the corresponding transfer of stearate from liposomes to BSA at 25°C; (○) indicates the respective equilibrium value from (A).

somes, incubated with the fatty acid anion just for 5 min, to BSA resulted in a relatively complex course of transfer passing through an expected minimum (Fig. 9). The final equilibrium distribution between liposomes and BSA was the same as after the long-term incubation. Fitting equation 3 with n = 3 to the data points of transfer after short-term incubation of liposomes with heptafluorostearate revealed two phases of transfer. The first complex phase represents the transfer of >70% of heptafluorostearate from liposomes to BSA and the following slow phase, characterized by a half-life of about 1 h, indicates transfer of a small amount back to the liposomes to reach the final equilibrium. The different amounts of heptafluorostearate transferred after short- or long-term incubation during the fast and the slow phases as well as the direction of transfer during the slow phases proved the identity of the slow transfer processes (Figs. 7 and 9) with the transversal movement of heptafluorostearate.

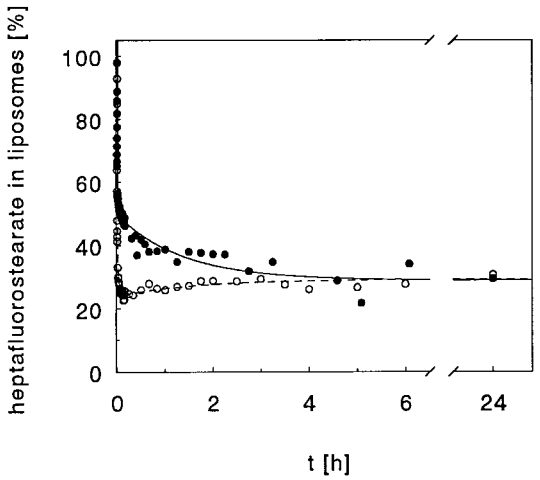

Fig. 9. Transfer course of heptafluorostearate from preincubated liposomes to BSA at 25°C. To a stirred suspension of 2 ml of empty liposomes, corresponding to a cholesterol concentration of 63 μm, 7 μl of a 4.5 mm solution of heptafluorostearate in ethanol was added. After incubation for (· · · ·, ○) 5 min or (—, ●) 24 h at 25°C, 0.8 ml of the liposomal suspension was added to 2 ml of a 2.1 μm BSA. Taking into account the dilution factor of 0.714, the total final concentrations of cholesterol, heptafluorostearate, and BSA were 9, 4.5, and 1.5 μm, respectively. Transformation of the measured fluorescence intensities into the depicted percent of liposomal heptafluorostearate was performed as described in Materials and Methods using the appropriate control curve as baseline. For the sake of clarity, between 10 and 600 s only every 20th data point is drawn. The curves represent the best fits to equation 3 with n = 3.

Analysis of transversal movement of heptafluorostearate

The transfer curves of heptafluorostearate exhibiting two phases, a fast and a relatively slow one (Fig. 7, solid line), are better described by 3 than by 2 exponential functions, as judged by the runs test (24) and the Akaike criterion (25). Consistent analysis of the curves of heptafluorostearate transfer from liposomes to BSA (Fig. 7, solid line), that is n = 3 in equation 3, reveals that the fast phase of transfer is better described by 2 than by 1 exponential function, whereas the relatively slow one is satisfactorily describable by 1 exponential function (**Table 2**). Expressed in % of total heptafluorostearate present, the amplitudes $A_{D,i}$ belonging to the three functions disclose

TABLE 2. Parameters of transfer of heptafluorostearate from liposomes to BSA at 25°C (n = 9)

Exponential Function i	Rate Constant k_i	Half-life $\tau_{1/2,i}$	Amplitude $A_{D,i}$
	h^{-1}	h	%
I	$(8.3 \pm 1.9)\cdot 10^{2}$	$(8.4 \pm 1.6)\cdot 10^{-4}$	43.1 ± 2.5
II	$(1.7 \pm 0.1)\cdot 10$	$(4.1 \pm 0.2)\cdot 10^{-2}$	12.6 ± 1.2
III	$(3.0 \pm 0.5)\cdot 10^{-1}$	2.3 ± 0.4	22.7 ± 2.5

Curves were fitted with equation 3 taking liposomes as donor compartment ($A_{D,i}$). The obtained rate constants k_i were converted into the corresponding half-lifes $\tau_{1/2,i}$ according to the relation $\tau_{1/2} = \ln 2/k$.

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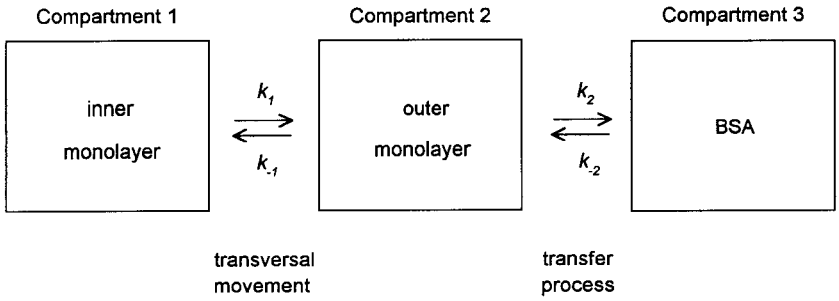

Fig. 10. Schematic representation of the three-compartment model. Compartments 1 and 2 represent the inner and the outer monolayer of the liposomal membrane and compartment 3 represents BSA. The rate constants k_1 and k_{-1} describe the transversal movement between the inner and the outer monolayer, k_2 and k_{-2} describe formally the transfer process between the outer liposomal monolayer and BSA.

that the sum of heptafluorostearate transferred in the fast phase amounts to 55.7 ± 3.7%, whereas 22.7 ± 2.5% were transferred in the slow one. Conversion of the rate constants k_i of the 3 exponential functions into more familiar half-lifes $\tau_{1/2,i}$ shows (Table 2) that, compared with the short half-lifes of the 2 exponential functions describing the fast phase, the third function, assigned to the transversal movement (Fig. 7), has a relative long half-life of $\tau_{1/2,III} = 2.3 \pm 0.4$ h.

In order to estimate the rate constant of transversal movement of heptafluorosterate, its transfer curves were analyzed with the aid of a three-compartment model (**Fig. 10**), in which the inner and the outer monolayer of the liposomes are represented by the compartments 1 and 2, and BSA by compartment 3. The aqueous solution as a fourth separate compartment can be disregarded because it is of no relevance for the estimation of the transversal movement and it was not intended to analyze the complex fast phase of transfer.

Because the volumes of the three compartments in the model used for describing transfer of heptafluorostearate from liposomes to BSA are not equal, equations 5–8 have to be applied:

$$\Delta n_j^f = -k_i^{nc} \cdot n_{j(t)} \cdot \Delta t \qquad \textit{Eq. 5)}$$

and

$$\Delta n_j^r = k_{-i}^{nc} \cdot n_{j+1(t)} \cdot \Delta t \qquad \textit{Eq. 6)}$$

with:

$$k_i^{nc} = k_i \cdot \frac{k_i + k_{-1}}{k_i \cdot \frac{V_{j+1}}{V_j} + k_{-i}} \cdot \frac{V_{j+1}}{V_j} \qquad \textit{Eq. 7)}$$

and

$$k_{-i}^{nc} = k_{-i} \cdot \frac{k_i + k_{-i}}{k_i \cdot \frac{V_{j+1}}{V_j} + k_{-i}} \qquad \textit{Eq. 8)}$$

where k_i^{nc} = rate constant in compartment i in the forward direction

k_{-i}^{nc} = rate constant in compartment i in the reverse direction

$n_{j(t)}$ = number of moles in compartment j at time t

V_j = volume of compartment j.

Applying the three-compartment model (Fig. 10) and assuming that the rate constant for transversal movement of heptafluorosterate from the inner to the outer monolayer (k_1) equals the rate constant in the reverse direction (k_{-1}), the measured half-life of $\tau_{1/2,III} = 2.3 \pm 0.4$ h results in a rate constant for the flip-flop of $k_{FF,25} = 0.24 \pm 0.05\ h^{-1}$, which is in reasonable accordance with an estimation of the rate constant of flip-flop of physiological long-chain fatty acid anions at 24°C (11).

Transfer of heptafluorostearate from liposomes to BSA at 37°C

Because most studies of biological transport at the cellular level are carried out at 37°C, the suitability of heptafluorostearate for these studies has been shown by additional transfer studies at this temperature (**Fig. 11**). Under the experimental conditions used, in equilibrium 75 ± 5% of total heptafluorostearate were extra-liposomal and only 25 ± 4% were incorporated in liposomal membranes. The transfer curves exhibit 2 phases requiring 3 exponential functions for adequate mathematical description, as judged by the runs test (24) and the Akaike criterion (25). During the fast phase, 55 ± 2% of heptafluo-

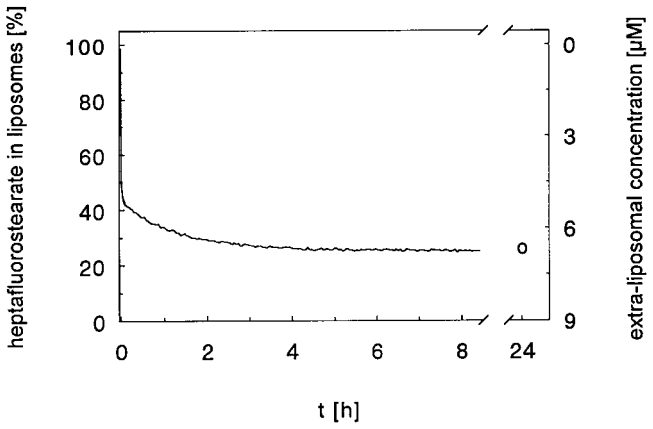

Fig. 11. Time dependency of the transfer of heptafluorostearate from liposomes to BSA at 37°C. Except for the temperature, all experimental conditions were the same as described in the legend of Fig. 7.

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rostearate was transferred at 37°C within 5 min. However, due to the high rates of transfer, it would be unwise to specify the parameters of the 2 exponential functions describing the fast phase of transfer. Only the parameters of the third exponential function, corresponding to the slow phase, could be estimated with reliability. From liposomes to BSA, 20 ± 4% of heptafluorostearate was transferred with a half-life $\tau_{1/2,III} = 0.9 \pm 0.1$ h. Using the three-compartment model (Fig. 10) with the assumption that the rate constants of transversal movement in both directions are equal, the measured half-life $\tau_{1/2,III}$ results in a rate constant for the flip-flop of heptafluorostearate at 37°C of $k_{FF,37} = 0.6 \pm 0.1\ h^{-1}$.

DISCUSSION

Heptafluorostearate has been designed as an isogeometric model compound of stearate (9), to facilitate the study of biological transport of long-chain fatty acid anions. Its mediated uptake into isolated hepatocytes has been demonstrated (10). The behavior of heptafluorostearate in comparison to stearate towards simple lipid mono- and bilayers, providing the basic knowledge to establish heptafluorostearate as a probe to differentiate between membrane transport of long-chain fatty acid anions by simple diffusion and by mediated processes, has not been analyzed.

The comparison of the surface pressure–area isotherms of the two pure fatty acid anions (Fig. 1) shows that stearate at pH 7.4 exhibits low compressibility and considerable cohesive forces between the film-forming molecules, whereas heptafluorostearate possesses high compressibility and low cohesive forces. The difference in the molecular areas of stearate at pH 7.4 and of heptafluorostearate mainly reflects the effect due to different pK_a values of the two fatty acids, because the larger molecular area of heptafluorostearate is in good agreement with that of stearate at pH 13 (Fig. 1) and is compatible with published data for stearate at pH ⩾12 and sodium dodecyl sulfate (27, 31). These results conclusively demonstrate that, even with regard to a pK_a shift of carboxylic acids >2 units when incorporated into monolayers (33–35), the intended difference in the acidic strengths of heptafluorostearic acid and stearic acid is sufficient to explain their behavior, and that steric effects of the fluorine atoms (30) have no influence. The difference in the molecular areas of heptafluorostearate and stearate at pH 7.4 is equalized by the excess of the other lipids, as shown by the virtually indistinguishable surface pressure–area isotherms of lipid mixtures containing 12.5 mol% of the fatty acid anions (Figs. 2A and B). This indicates that the electrostatic repulsions of the negatively charged carboxylate groups of long-chain fatty acid anions were reduced by their distribution in the lipid membrane, as shown with oleate (36). The differences of the molecular areas of heptafluorostearate and stearate in monolayers of the pure compounds are of minor importance in lipid mixtures, making heptafluorostearate suitable for comparative transfer studies.

Transfer of long-chain fatty acid anions from liposomes to BSA is best followed by continuous measurement using spectroscopic methods (16). Binding of long-chain fatty acid anions to BSA (37, 38) is accompanied by a fast conformational change of the protein (39, 40). Because the intrinsic fluorescence of BSA is very sensitive to this conformational change, its intrinsic fluorescence is also well suited for continuously following the transfer of long-chain fatty acid anions from liposomes to the extra-liposomal aqueous phase and vice versa. It must be emphasized that the investigation of the dependency of the intrinsic fluorescence of BSA on the total concentration of fatty acid anions, leading to the calibration plots (Figs. 3 and 4), was not intended to correlate the fluorescence intensity with the concentration of free fatty acid anion but only with the total concentration. The dependency of intrinsic fluorescence of BSA shows, for both fatty acid anions, a clear decrease with increasing concentrations, becoming more marked at higher temperatures (Fig. 3). The complex dependency of fluorescence quenching on total fatty acid anion concentrations was also observed for the binding of dodecyl sulfate to BSA (41) and represents no inconsistency with the linear one described previously (14), where a relatively high BSA concentration of 100 μm at room temperature was used.

In order to evaluate the suitability of heptafluorostearate as a model compound for the study of biological membrane transport of long-chain fatty acid anions, transfer of heptafluorostearate and stearate from liposomes to BSA was compared, using liposomes which contained phospholipids and cholesterol in a molar ratio of 100:40, similar to that in the plasma membrane of rat hepatocytes (42). The phospholipid POPG bearing a negative net charge was added to the lipid mixture to minimize unspecific interactions between the liposomes and the also negatively charged BSA at pH 7.4. As only about 7% of the membranes are not directly accessible, the interpretation of the transfer experiments is simplified and allows the application of a three-compartment model (Fig. 10) for analysis of the measurements. The diameter of the liposomes of 120–130 nm is so small that scattering effects were negligible, but so large that stress differences between the two monolayers of the liposomal membrane are practically absent.

The slight photodecomposition of BSA during long times of exposure to light accompanying continuous following of transfer of heptafluorostearate from liposomes to BSA was taken into account by control measurements (Fig. 6). The identical time course of the fluorescence intensity of pure BSA and of BSA in the presence of empty liposomes excludes an interference of the liposomes or of one of their components with the protein. The slight dependency of decrease of intrinsic fluorescence of BSA with time on the presence of heptafluorostearate was taken into account by using controls containing a concentration of fatty acid anion corresponding to that present in the extra-liposomal compartment after one half-life of the slowest process. The validity of this mode of analysis and the correctness of the transfer curves of heptafluo-

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rostearate resulting from continuously recording the fluorescence intensities was confirmed by discontinuous control measurements (Fig. 7).

The final equilibrium distributions of heptafluorostearate and stearate after transfer from loaded liposomes to BSA reached within 24 h and 3 min, respectively, at 25°C (Figs. 7 and 8B) differ slightly due to the higher affinity of BSA to stearate than to heptafluorostearate. The crucial difference of the transfer curve of heptafluorostearate to that of stearate, the presence of a relatively slow phase of transfer, may be explained by a slow rate of transversal movement being too fast to be detected for stearate with the experimental setup used (1, 2, 4, 5). Assuming the same distribution of the fatty acid anion as the phospholipids in both monolayers of the liposomes, the fast portion of transfer corresponds well to the amount of heptafluorostarate initially present in the outer monolayer of about 50% and the slow one to the amount being transferred to BSA subsequent to the slow transversal movement of heptafluorostarate from the inner to the outer monolayer. This interpretation was confirmed conclusively by comparison of short- and long-term incorporation of heptafluorostearate into empty liposomes (Fig. 9). Short-term incubation of liposomes with heptafluorostearate, not allowing a significant amount of the fatty acid anion to reach the inner monolayer of the liposomal membrane, resulted in >70% of heptafluorostearate fastly transferable to BSA. By contrast, long-term incubation, enabling heptafluorostearate to get into the inner monolayer by slow transversal movement, made only about 50% of the fatty acid anion accessible to fast transfer. The different half-lifes of the transversal movement of heptafluorostearate in the absence (Fig. 7) and presence (Fig. 9) of ethanol are easily explained by the interaction of the alcohol with the liposomal membrane (43). The minimum in the transfer curve of heptafluorostearate after short-term incubation may be explained by an uncomplete equilibration between outer and inner monolayer within 5 min requiring the supply of heptafluorostearate back from BSA to liposomes to attain the final equilibrium.

Whereas the third exponential function in the equations 3 and 4 with n = 3, describing the transfer of heptafluorostearate, corresponds to its transversal movement, the assignment of the two exponential functions, required for description of its fast phase of transfer, to a simple physical process is uncertain. Methodological artefacts originating from impurities of the lipid components and from transfer of lipid components other than fatty acid anions can be excluded. The presence of a substantial portion of free heptafluorostearate compared to total can be ruled out due to its low solubility and its equilibrium constant K_{eq} of $5 \cdot 10^6$ (W. Schmider and G. Kurz, unpublished results). The involvement of different binding sites of BSA is probably not the reason for the complex fast phase, because in transfer from liposomes containing only 2 mol% of heptafluorostearate to BSA (data not shown) the same complexity was observed. By exclusion of the aspects discussed above, interferences of changes in the liposomal membrane with the transfer of the fatty acid anions are more likely. The influence of the changing electrical field during transfer of heptafluorostearate is expected to be relatively small (44, 45), and the heterogeneity in liposome diameter is too small to account for the large differences between the corresponding rate constants. An inhomogeneity in the distribution of heptafluorostearate in coexisting subphases of the liposomal membrane may account for the complex transfer course (46, 47), even if a clustering of fatty acid anions may be ruled out due to the results of the monolayer experiments (Fig. 2, and ref. 48). The asymmetric distribution of heptafluorostearate between inner and outer monolayer of the liposomal membrane generated within the first sec of transfer may produce stress differences between the lipids of the two opposing monolayers. Stress differences between the monolayers of the liposomal membrane, induced by osmotic pressure, were also postulated to explain the biphasic transversal movement of phospholipids, the one with the longer half-life coinciding with the real flip-flop process (49). Whereas these stress differences should be equalized by the rapid flip-flop of cholesterol (50, 51) the asymmetric distribution of heptafluorostearate persists and may become apparent in a retardation of transfer of heptafluorostearate from the outer monolayer to BSA and/or in an acceleration of its initial transversal movement. Whatever the reasons for the two exponential functions are, the fast phase of transfer is finished after 10 min and subsequently transversal movement of heptafluorostearate occurs by its real slow rate characterized by a half-life of about 2.3 h, the actual mechanism of which is presently under investigation. The absence of any slow process in the transfer of stearate is to be expected for a long-chain fatty acid, the transversal movement of which occurs with a half-life in the range of msec (4) and demonstrates clearly that the fundamental difference in the transfer of stearate and heptafluorostearate results from their different transversal movement due to the different pK_a values of their corresponding fatty acids.

Despite the enormous efforts and the application of various sophisticated methodologies, considerable disagreement persists regarding the mechanism by which long-chain fatty acid anions enter cells (6, 43, 52). The transfer studies presented suggest, in accordance with previous results (4, 5), that dissociation from the membrane is the rate-limiting step in membrane permeation of physiological long-chain fatty acid anions. Because mediated uptake of stearate into freshly isolated hepatocytes was unequivocally demonstrated (10), it might be concluded that the dissociation step has to be catalyzed. Taking into account micro-reversibility in mediated transport processes and the fact that heptafluorostearate is taken up by the same transporters as stearate (10), a reasonable model of biological membrane transport of fatty acid anions may be that the transporter catalyzes dissociation of physiological fatty acid anions at both sides of the membrane and that it stays in contact with its substrate during transversal movement. In the case of heptafluorostearate, it came out that the transporter catalyzes not only the dissociation of the fatty acid anion from the membrane but also its transver-

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sal movement. Thus, heptafluorostearate is an ideal probe for the study of mediated transport of long-chain fatty acid anions, because it shows only the intended deviation in its behavior compared to stearate and requires mediated processes for effective transport through biological membranes. Uptake studies using heptafluorostearate allow a fast and definite decision as to whether fatty acid anions are taken up by mediated transport into the cells of interest.

The authors express their gratitude to Dr. W. Deck (Institut für Anorganische und Analytische Chemie der Universität Freiburg, Germany) as well as to Dr. D. Hunkler (Institut für Organische Chemie und Biochemie der Universität Freiburg) for measuring and interpreting the NMR spectra, and to Dr. J-U. Weidner (Institut für Physikalische Chemie der Universität Freiburg) for assistance in the solution of differential equations. They are grateful to PD Dr. A. Seelig (Biozentrum der Universität Basel, Basel, Switzerland) and T. Alt for the assistance in monolayer experiments, Prof. Dr. R. E. Schubert (Pharmazeutisches Institut der Universität Freiburg) for the opportunity to measure size distributions of liposomes and Prof. Dr. B. Volk (Neurozentrum des Universitäts-Klinikums der Universität Freiburg) to perform fluorescence measurements. The authors thank K. Müller (Institut für Pharmazeutische Technologie und Biopharmazie der Universität Marburg, Germany) for experimental assistance. This study was supported by a special program of the Universität Freiburg and of the Universitäts-Klinikum der Universität Freiburg.

Manuscript received 26 May 1998, in revised form 29 October 1999, and in re-revised form 8 February 2000.

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