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# Sheep Erythrocyte Membrane Binding and Transfer of Long-Chain Fatty Acids

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**Abstract.** We have studied binding and membrane transfer rates of unsaturated long-chain fatty acids in sheep red cells, as previously done for human red cells, in order to elucidate the transport mechanism. Observed differences must be assigned to the different composition of the membrane in the two species.

Equal surface areas of the membranes of the two species have similar binding capacities and affinities for palmitic-, linoleic-, oleic- and arachidonic acid at 37°C. The competitive bindings of linoleic- and arachidonic acid as well as the distribution of bound arachidonic acid on the two sides of the membrane are not different in the two species. However, the rate constants for membrane transfer in sheep are less than half of those measured previously for human ghosts. This finding is confirmed by the exchange efflux kinetics of ghosts containing albumin-bound fatty acid. Studies of sheep ghost membranes with oleic-, arachidonic- and linoleic acid reveal a proportionality between the membrane transfer rate constants and the number of fatty acid double bonds, as found previously for human ghost membrane, and the effect of double bonds is in harmony with a large negative activation entropy for diffusion through the membrane. The established replacement of lecithin by sphingomyelin with a low unsaturation fatty acid index in sheep membranes probably causes a lower transversal lipid phase fluidity. Double bonds diminish the flexibility of hydrocarbon chains and thus the large negative activation entropy of diffusion across the membrane. The smaller transfer rate constants of the three unsaturated fatty acids in sheep membranes support the hypothesis that the transfer is diffusion in protein defined annular lipid domains and not carrier mediated.

**Key words:** Palmitic acid — Oleic acid — Linoleic acid — Arachidonic acid — Sheep erythrocyte ghosts — Transporting elements — Transport kinetics — Fatty acid transport — Transport rate constants

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## Introduction

For several years two mutually exclusive mechanisms have been suggested for membrane binding and transfer of long-chain fatty acids (FAs). Studies of metabolizing cells and their membranes suggest a protein carrier mechanism [*see* Table 1 [2], 15, 27], whereas studies of phospholipid bilayers suggest that the lipid bilayer alone mediates transfer [20, 21, 23, 29]. We have suggested a third possibility according to studies of FA binding and transfer through resealed human erythrocyte membranes (ghosts) [6, 7, 8, 9, 10, 11, 12].

Just as other cell membranes, the membrane of red cells has limited binding capacities for monomer FA, the concentration of which is defined by the three equivalent high affinity sites of albumin. These limited capacities do not necessarily imply protein carrier bindings. In fact, such an implication is doubtful, because FA have different binding capacities distributed differently on the inside and outside of the membrane. Membrane proteins however may be implicated in two ways in defining binding capacities. Small annular domains of phospholipids exchange rapidly with the bulk lipids and such domains may be different for different FA by adapting to different hydrocarbon chains. Furthermore, membrane proteins may provide the cationic charge required for initial electrostatic binding of the anionic FA just as the cationic sites of the high-affinity sites of albumin [5]. Lipid phase transfer of FA probably requires protonation of the anion to a neutral form as seen for other charged molecules [16]. A further support for lipid channelling was the new finding that the rate constants for unsaturated FA transfer between the two binding sites of the

membrane were proportional to the number of FA double bonds [12]. Thus our ghost studies suggest a lipid phase permeation through a number of specific constitutive elements.

Sheep red cells are smaller than human red cells and contain sphingomyelins rather than lecithins, with a low fraction of unsaturated fatty acids. As this difference is expected to produce a greater microviscosity [14] the lipid channelling hypothesis would suggest a much smaller membrane transfer rate for sheep cells than for human cells. The present data show that this is indeed the only measured difference between cells from the two species regarding binding and transfer of FA.

## Materials and Methods

[5,6,8,9,11,12,14,15-$^3$H] Arachidonic acid (20:4), sp. act. 200 Ci/mmol, [9,10-$^3$H] Palmitic acid (16:0), sp. act. 54 Ci/mmol, [1-$^{14}$C] Linoleic acid (18:2), sp. act. 58 mCi/mmol and [9,10-$^3$] Oleic acid (18:1), sp. act. 4.2 Ci/mmol were all purchased from Amersham International, Amersham, England. All unlabeled fatty acids were purchased from Sigma. Labeled as well as unlabeled FAs were purified monthly by column chromatography, ascertaining the elution pattern of a single component. The scintillation fluid Ultima Gold was from Packard Instrument, (Downers Grove, IL). Bovine serum albumin (BSA) was obtained from Behring Institute, Germany and defatted. The sheeps were Texel/Gotland.

### Experimental and Theoretical Methods

#### *Preparation of Sheep Ghosts and Buffer with FA-BSA Complexes*

0.05% (7.5 μM) BSA-filled sheep ghosts and BSA-free sheep ghosts were prepared by hypotonic hemolysis at 0°C in media with or without BSA [7]. After resealing of ghosts at 38°C for 45 min, the pink ghosts were washed three times with 165 mM KCl, 2 mM phosphate buffer, pH 7.3 containing 0.02 mM EDTA/EGTA (1:1). Finally, the ghosts were washed in buffer containing the appropriate amount of BSA. The amount of [$^3$H] or [$^{14}$C] FAs and of unlabeled FAs to give 1–5 μCi/ml and final molar ratios of FA to BSA ($\nu$) from about 0.25 to 1.7 was deposited on glass beads as described previously [10]. These were shaken with the buffer containing BSA for 15 min at room temperature.

#### *Labeling of Ghosts and Uptakes of FAs*

Sheep ghosts were packed by centrifugation 7 min (at 36,400 × $g$). One volume of ghosts was equilibrated at the appropriate temperature with 1.5 volumes of the solution containing radioactive FA. The BSA containing buffer was removed and counted ($C_a$ dpm ml$^{-1}$) in order to calculate final $\nu$ values, as $C_a/(S_a \times [\text{BSA}])$, where $S_a$ is FA specific activity in dpm nmoles$^{-1}$.

#### *Fatty Acid Uptake by Ghosts Equilibrated with Fatty Acids Bound to BSA in the Molar Ratio $\nu$*

The amount of FA bound to the membrane ($M$) in nmol g$^{-1}$, and final $\nu$ values are calculated on the basis of $S_a$ and of counting rates in the labeling solutions before and after equilibration with ghosts. Alternatively, $M$ was calculated on the basis of weighed washed samples of packed ghosts, taking into consideration the extracellular volume trapped in packed ghosts. We have shown [12] for 5 different FA that the uptake by 1 g human red cell ghosts ($M$) with three binding sites on BSA is described by the equation:

$$M = C\,\nu/[\nu + (K_{dm}/K_d)(3\text{-}\nu)] \tag{1}$$

where $C$ (nmol g$^{-1}$) is the binding capacity, $K_{dm}$ and $K_d$ are equilibrium dissociation constants of FA bound to ghosts and BSA, respectively. A linearization of Eq. 1 is:

$$(\nu/(3-\nu))/M = (1/C)(\nu/(3-\nu)) + (K_{dm}/K_d)(1/C) \tag{2}$$

the capacity and the ratio of equilibrium constant can be estimated by linear regressions.

#### *Competitive vs. Noncompetitive Bindings of Two FA with Different Binding Capacities*

Fatty acid uptakes are measured in double isotope studies of two FAs bound to albumin in various $\nu$ values. The present studies with sheep ghosts are carried out as previously described for human ghosts [7, 8, 9, 10, 11, 12]. When $FA_1$ and $FA_2$ share the smallest binding capacity of $FA_1$, we calculate the uptake of the two fatty acids ($m_{1c}$ and $m_{2c}$) in competition by two equations analogous to equations of competitive enzyme inhibition substituting the initial velocity and maximum velocity by the uptake of FA ($m$) and $C$, respectively [12]. The equations are derived in a previous paper (Eqs. 6 and 7 in [12]).

$$m_{1c} = C_1/[(K_{dm1}/K_{d1})(3\text{-}\nu_1\text{-}\nu_2)/\nu_1 + (K_{dm1}K_{d2}\nu_2)/(K_{d1}K_{dm2}\nu_1) + 1] \tag{3}$$

$$m_{2c} = C_1/[(K_{dm2}/K_{d2})(3\text{-}\nu_1\text{-}\nu_2)/\nu_2 + (K_{dm2}K_{d1}\nu_1)/(K_{d2}K_{dm1}\nu_2) + 1] \tag{4}$$

From Eq. 3 and Eq. 4 $m_{1c}$ and $m_{2c}$ can be calculated exclusively from estimated parameters.

In addition $FA_2$ alone is bound noncompetitively by the binding capacity ($C_2 - C_1$). This specific binding is called $m_{2sp}$ and it is calculated according to the previously published Eqs. 8 [12].

$$m_{2sp} = (C_2 - C_1)/(1 + (K_{dm2}/K_{d2})(3\text{-}\nu_1\text{-}\nu_2)/\nu_2) \tag{5}$$

In Table 2 the bindings of linoleic acid (18:2) are equal to $m_{2c} + m_{2sp}$. In the case of noncompetitive bindings, the uptake of $FA_1$ is calculated by the following equation:

$$m_1 = C_1/[1 + (K_{dm1}/K_{d1})(3\text{-}\nu_1\text{-}\nu_2)/\nu_1] \tag{6}$$

and the analogous equation for $FA_2$.

In addition we calculate ratios of measured- to theoretical uptakes ($M/m$).

#### *Exchange Efflux Experiments*

Labeled and washed ghosts were packed by centrifugation at 0°C for 10 min at 10,000 × $g$ in plastic tubes (i.d. 3 mm). The supernatant was removed by cutting the tube just below the interface and the ghosts (about 200 μl) were injected into 35 ml stirred 165 mM KCl, 2 mM phosphate buffer pH 7.3 containing 0.02 mM EDTA/EGTA (1/1) and unlabeled FA bound to BSA. The efflux media were also prepared by the glass-bead technique (*see above*), but up to 2–3 g of glass beads were used for disposition of FA.

Serial sampling of cell-free extracellular medium was done as

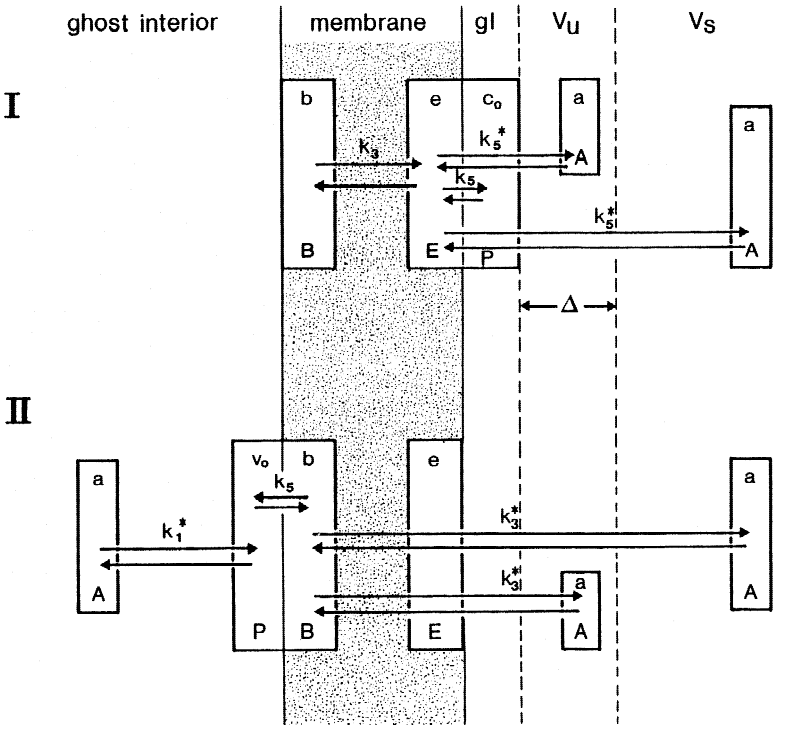


**Fig. 1.** The compartment models used to account for efflux of tracer-fatty acid from ghosts in nonisotopic equilibrium with the medium. I: Ghosts without serum albumin. II: Ghosts with serum albumin. Arrows indicate unidirectional fatty acid fluxes. $k_1^*$, $k_3$, $k_3^*$, $k_5$ and $k_5^*$ are first order rate constants of fluxes between adjacent compartments. Lower case letters a, b, c, $c_o$ and $v_o$ refer to the amount of labeled fatty acids per ml packed ghosts and upper case letters A, B, E, P to the amount of unlabeled fatty per ml ghosts, P is in the text call [FA]. $V_u$ is unstirred volume around ghosts with the depth Δ and $V_s$ the stirred volume around ghosts. gl is a 10 nm deep glycocalyx space. Reprinted with permission from *Acta Physiologica Scandinavica* 1995. *154:*253–267.

described previously [7] and the radioactivity of 400 μl samples was measured by scintillation counting. Figure 1 presents the models used in previous studies with resealed human red cell membranes to account for FA tracer exchange efflux kinetics for which previous publications present derivations of the applicable equations [8, 9, 10]. In this paper only final, data applicable, equations are presented with reference to previous relevant papers. The biexponential efflux kinetics predicted by the model I (three-compartment closed system) has the solution:

$$(1 - y/y_\infty) = K_1\, e^{-\alpha t} + K_2\, e^{-\beta t} \tag{7}$$

where $y/y_\infty$ is the fraction of tracer in the large medium volume at any time ($t$) after the addition of the labeled ghosts. $K_1 + K_2 = 1$ and $K_2$ is the value suggested for the final part by graphic analysis of the data and fitted to obtain maximum correlation coefficient $R$ by nonlinear regression analyses using STATGRAPHIC version 5. Three model parameters: $B/E$, $k_3$ and $k_5^*$ (*see* Fig. 1) are defined by the kinetics according to the equations.

$$k_5^* = \alpha + \beta - \alpha\beta/(\alpha K_1 + \beta K_2) \tag{8a}$$

$$k_3 = \alpha\beta/k_5^* \tag{8b}$$

$$1 + B/E = k_5^*/(\alpha K_1 + \beta K_2) \tag{8c}$$

From $k_5^*$ it is possible to calculate $K_1$, the dissociation rate constant of BSA bound FA, according to the theoretical effect of the unstirred layer surrounding ghosts by Eq. (2.4) in [9], which may be expressed more simply as

$$k_1 = \left[\frac{\frac{k_5^* E}{[FA]} - 1/R_D}{S\sqrt{D}}\right]^2 \left(\frac{[FA]}{\nu\,[BSA_y]}\right) \tag{8d}$$

where $[BSA_y]$ is the BSA concentration in the efflux medium and $D$ is the diffusion coefficient of FA in $cm^2\ sec^{-1}$. $E$ is nmoles FA in the membrane outer layer of ghosts, $S$ is surface area of ghosts ($cm^2$) and $1/R_D$ is diffusion conductivity of the unstirred volume surrounding ghosts (ml $sec^{-1}$), all three parameters normalized to 1 ml ghosts.

## *Tracer Exchange Efflux Kinetics from Ghosts Containing 7.5 μM BSA*

The kinetics are essentially biexponential after an initial release of tracer bound by the outer membrane leaflet ($E$). The two rate coefficients of the biexponential kinetics are called δ and γ [8, 9, 10] and a coefficient $\delta^* = \delta/(1 + \delta/\gamma)$ can be calculated on the basis of the observed δ and γ. A theoretical δ*(*theor*) is determined by the sequence of steps: FA release from the internal FA pool (FA bound to internal BSA, $k_1^*$) — FA binding to the FA pool at the inner membrane surface ($B$, $k_5$) — FA flow through the outer membrane surface ($E$) to the external FA pool (FA bound to extracellular BSA, $k_3^*$) and the equation which according to the model II (a three compartment approximation) accounts for this parameter is:

$$\delta^*(theor) = \frac{Q}{1/k_5 + 1/k_3^* + Q(1/k_3^* + 1/k_1^*)} \tag{9}$$

where $Q$ is the ratio of FA in transport pool $B$ to FA bound to BSA within ghosts, $k_3^*$ the rate constant of unidirectional flow from $B$ to BSA in the medium through the $E$ compartment (derived from Eq. A14 [10]), $k_1^*$ the integrated effective mean dissociation constant of FA-BSA complex within ghosts (derived from $k_1$ by Eq. A12 [10]) and $k_5$ the rate constant of unidirectional flow from $B$ to the adjacent water-phase (*see* Fig. 1 model II). The parameter values require information of: firstly membrane binding ($M$) and distribution on inside and outside ($B/E$), secondly of the rate constant of transfer from inside to outside binding ($k_3$) and the rate constant $k_5^*$ in order to convert $k_3$ to $k_3^*$ and thirdly of the dissociation rate constant of BSA binding ($k_1$). Now all

**Table 1.** Measured uptake at 37°C of four fatty acids (M in nmoles $g^{-1}$ packed sheep ghosts) at various values of fatty acid/albumin molar ratio's ($v$) and the uptakes by sheep ghosts compared to uptake by human ghosts (in nmoles FA $g^{-1}$) calculated according to Eq. 1 (sheep/human)

| Fatty acids | $v$ | M nmoles FA/g ghosts | Sheep/Human |
|---|---|---|---|
| Linoleic acid | 1.72 | 8.28 | 1.54 |
| | 0.66 | 4.07 | 1.44 |
| | 0.47 | 2.78 | 1.39 |
| | 0.23 | 1.29 | 1.30 |
| | | | 1.42 ± 0.06 |
| Oleic acid | 1.02 | 11.84 | 1.42 |
| | 0.48 | 5.55 | 1.50 |
| | 0.31 | 3.08 | 1.41 |
| | 0.155 | 1.49 | 1.40 |
| | | | 1.43 ± 0.03 |
| Palmitic acid | 0.24 | 8.20 | 1.53 |
| | 0.453 | 15.00 | 1.62 |
| | 0.126 | 4.00 | 1.33 |
| | 0.486 | 15.20 | 1.55 |
| | | | 1.51 ± 0.07 |
| Arachidonic acid | 0.46 | 3.30 | 1.18 |
| | 1.09 | 9.20 | 2.12 |
| | 0.77 | 5.53 | 1.50 |
| | 0.72 | 4.75 | 1.32 |
| | | | 1.53 ± 0.24 |

the parameters which form part of the Eq. 9 are calculated from $M$, $B/E$, $k_3$, $k_5^*$ and $k_1$. The equations are described in detail previously [8, 9, 10]. The unstirred volumes within the ghosts and the adjacent medium have predictable effects on the virtual values of the rate constants $k_3$ and $k_1$ [8, 9, 10]. In calculation of δ*, the factor $1/k_5$ is of little significance because $k_5$ is much greater than $k_3^*$ and it is therefore neglected. After $Q$, $1/k_3^*$ is the most important value and is mainly determined by the true $k_3$ obtained by tracer exchange efflux from ghosts without BSA. It should be noted that the true radius of sheep ghosts and the surface area of 1 g sheep ghosts is used in the calculation of $k_1^*$ and $k_3^*$ from the true constants.

### *Statistics*

SES of values computed by complex expressions are evaluated by numeric differentiations and SES of the components, neglecting covariations [4].

## Results

### Binding Capacities and Affinities of Sheep Ghost Membranes

Table 1 shows the uptakes of four FA by sheep ghosts equilibrated with FA bound to BSA in the indicated molar ratios $v$. We presume that the binding capacities of ghosts are proportional to the size of the membrane area. As this is proportional to the radius squared, whereas the number of cells per g is inversely proportional to the radius in the third power and the total area of ghost membrane in 1 g packed cells is inversely proportional to the cell radius. The diameters of sheep and human red cells are 5.2 and 7.9 μm, respectively [13]. Thus, we presume that the binding capacity of 1 g sheep ghosts is 1.5-fold greater than of 1 g human ghosts. The prediction is confirmed (Table 1) by comparing the FA uptakes by sheep membranes with uptakes calculated by Eq. 1 for human ghosts cells using the capacities $C$ of 1 g ghosts and the values of $K_{dm}/K_d$ known for human ghosts. On average the uptake ratio by 1 g ghosts from the two species are not significantly different from the expected value of 1.5, demonstrating that the binding capacities per cell membrane area are indistinguishable as are the different ratios $K_{dm}/K_d$ of four FA. Thus $K_{dm}$ of human ghost binding is valid for sheep ghosts as well.

### Competitive Membrane Bindings of Two FA, Linoleic Acid and Arachidonic Acid

The equilibrium uptake by sheep ghosts has been studied in double isotope experiments with $^3$H-arachidonic acid and $^{14}$C-linoleic acid. Different values of $v$ are used with the sum being between 0.7 to 1.8. Table 2 shows the actual uptake of the two FA and the uptake compared to uptake calculated according to Eq. 6 assuming no competition and according to Eqs. 3, 4 and 5 assuming competition. The two FA mutually diminish the bindings of each other approximately 25% and simple competitive binding to the capacity of arachidonic acid together with noncompetitive binding of linoleic acid in the residual capacity account for the data. Thus the competition conditions are exactly as seen for human ghosts [12].

### Exchange Efflux of Arachidonic Acid from BSA-Free Ghosts to BSA in Media

The data are analyzed as previously for human ghosts according to model I. The expected biexponential time course according to Eq. 7 appears in the semilogarithmic plot (Fig. 2*A*). Nonlinear regression analysis, assuming a biexponential time course also defines the rate coefficients α and β as well as $K_2$ fairly well. Thus the values of the model parameters $B/E$, $k_3$ and $k_5^*$ can be found according to Eqs. 8*a,b,c*. The distribution ratio of AR in the sheep red cell membrane ($B/E$) and $k_1$ calculated from $k_5^*$ are not significantly different from the values obtained with human membrane, whereas the rate constant of unidirectional arachidonic acid flow from $B$ to $E$ ($k_3$) is 0.15 ± 0.02 $sec^{-1}$ compared to 0.39 ± 0.03 $sec^{-1}$ for human ghosts.

**Table 2.** Uptakes ($M$ and $m$) of $^{14}$C-linoleic acid (18:2, $FA_2$) and $^{3}$H-arachidonic acid (20:4, $FA_1$) by sheep ghosts in equilibrium with the fatty acids (FA) bound to bovine serum albumin (BSA) at various molar ratios of fatty acids to BSA ($v$)

| $v$ $FA_2$ | $FA_1$ | $M_2$ Uptake ($M$) nmolesFA/g | $M_1$ | $M_2/m_2$ Uptake ÷ competition nmolesFA/g | $M_1/m_1$ | $M_2/(m_{2c} + m_{2sp})$ Uptake + competition nmolesFA/g | $M_1/m_{1c}$ |
|---|---|---|---|---|---|---|---|
| 18:2 | 20:4 | 18:2 | 20:4 | 18:2 | 20:4 | 18:2 | 20:4 |
| 0.79 | 0.64 | 4.5 | 4.6 | 0.70 (0.07) | 0.81 (0.12) | 0.97 (0.15) | 0.95 (0.14) |
| 1.01 | 0.80 | 5.5 | 5.63 | 0.63 (0.08) | 0.87 (0.12) | 0.91 (0.11) | 1.03 (0.15) |
| 0.41 | 0.31 | 2.2 | 2.3 | 0.76 (0.10) | 0.65 (0.11) | 0.92 (0.11) | 0.73 (0.14) |
| 0.50 | 0.40 | 2.8 | 2.64 | 0.76 (0.12) | 0.63 (0.12) | 0.956 (0.17) | 0.70 (0.15) |

$M_1$ and $M_2$ are experimental measured uptakes of $FA_1$ and $FA_2$. $m_1$ and $m_2$ are uptakes calculated according to Eq. 6 and $m_{1c}$ and $m_{2c} + m_{2sp}$ according to Eqs. 3, 4 and 5. The two FA mutually diminish the bindings of each other some 25% calculated on basis of a 1.5-fold greater binding capacity of 1 ml packed sheep ghosts than of 1 ml packed human ghosts, with identical affinities, $K_{dm}$s. Simple competitive bindings of 18:2 to sites of 20:4 binding capacity account for the diminished bindings. Figures in parentheses are SES.

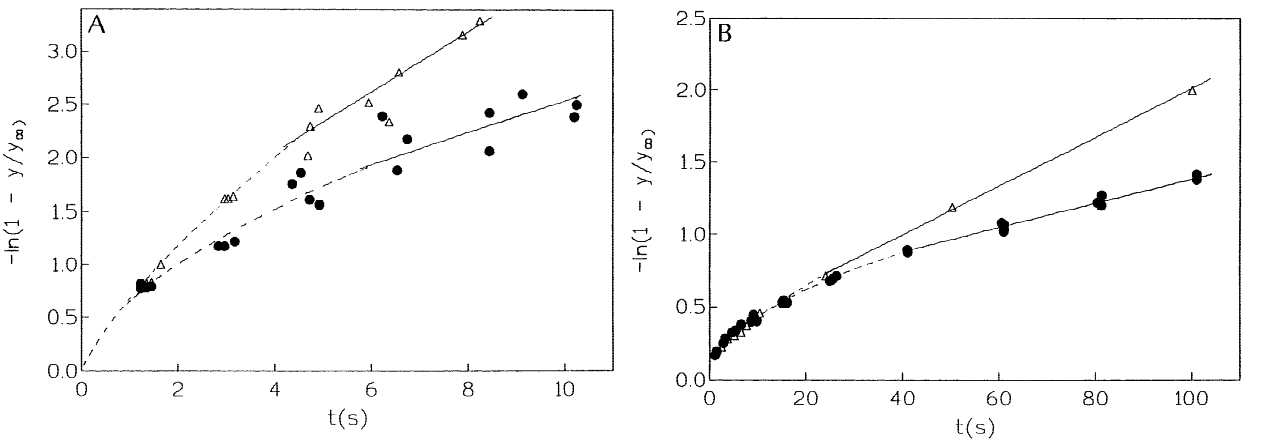


**Fig. 2.** (*A*) Exchange efflux kinetics of arachidonic acid (20:4) from BSA-free sheep ghosts at 0°C, $v = 0.6$ ● and from BSA-free human ghosts at 0°C, $v = 0.6$ Δ. The extracellular medium contains 0.05% (7.45 μM) BSA. Solid curves show the nonlinear regressions (STATGRAPHIC 5) fitting the data according to Eq. 6 (*see* methods) $y/y_\infty$ is the ratio of time dependent amount of tracer fatty acid in the medium to that at infinite time. (*B*) Exchange efflux kinetics of arachidonic acid (20:4) from 0.05% BSA-filled sheep ghosts at 0°C, $v = 0.6$ ● and from 0.05% BSA-filled human ghosts at 0°C, $v = 0.6$ Δ into a medium of 0.05% BSA. Solid curves show the nonlinear regressions (STATGRAPHIC 5) fitting the data according to the solution of the biexponential efflux kinetics predicted by model II (a three-compartment approximation) (*see* Fig. 1). $y/y_\infty$ is the same as above.

### Exchange Efflux of Three Unsaturated FA from Sheep Ghosts Containing 7.5 μM BSA to Media of 7.5 μM BSA

Figure 2*B* shows the tracer exchange efflux from sheep and human ghosts of arachidonic acid in similar experiments. These experiments provide determination of the two rate coefficients γ and δ by nonlinear regression analyses. Thus we get a value of $\delta^* = \delta/(1 + \delta/\gamma)$ equal to 0.00865 ± 0.00040 $sec^{-1}$ not significantly different from the expected theoretical value $\delta^*(theor) = 0.00943$ ± 0.00106 $sec^{-1}$ calculated by Eq. 9 for which we have all the parameters from tracer exchange efflux from ghosts without BSA. This confirms that the use of $k_3 = 0.15$ $sec^{-1}$ is valid in membrane transfer of arachidonic acid between the two pools of BSA bound FA. Figure 3*A* shows that the efflux of linoleic acid in similar experiments with sheep ghosts is slower than with human ghosts. In this case, we get the $k_3$ value by fitting the theoretical $\delta^*(theor)$ (Eq. 9) to the experimental value $\delta^* = \delta/(1 + \delta/\gamma) = 0.0034$ $sec^{-1}$. This is obtained by $k_3 = 0.065$ $sec^{-1}$. Figure 3*B* shows that the efflux kinetics of oleic acid from sheep and human cells are not different. Fitting the theoretical to the experimental δ* gives the

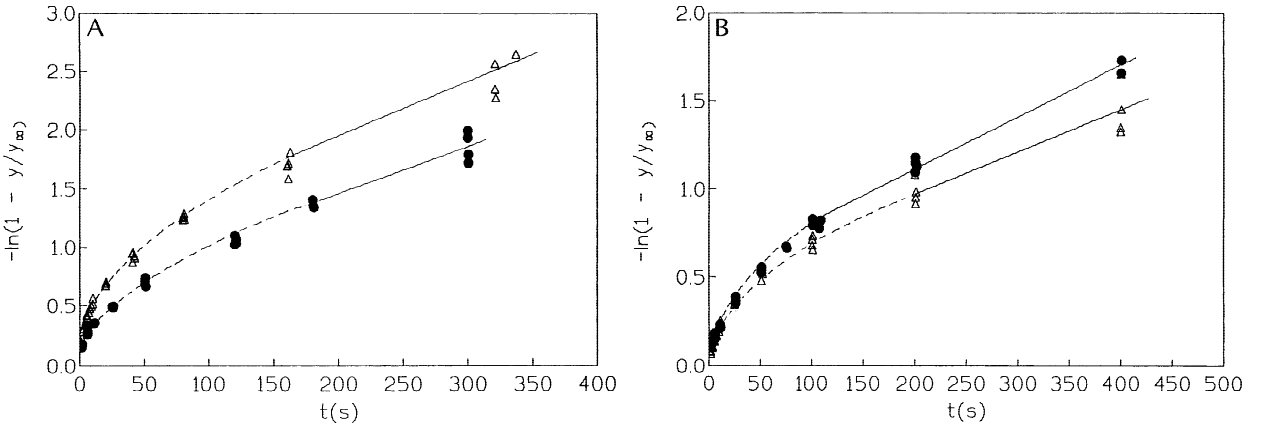


**Fig. 3.** (*A*) Exchange efflux kinetics of linoleic acid (18:2) from 0.05% BSA-filled sheep ghosts ● and human ghosts Δ at 0°C, $v = 0.65$. (*B*) Exchange efflux kinetics of oleic acid (18:1) from 0.05% BSA-filled sheep ghosts ● and human ghosts Δ at 0°C, $v = 0.65$.

small $k_3$ value of 0.035 $sec^{-1}$ in the case of sheep ghosts. The effect of higher capacity and $Q$ per ml ghosts is counterbalanced by this $k_3$ much lower than the $k_3$ value of human ghosts (Table 3). In the case of palmitic acid, experiments do not permit a determination of a precise value of $k_3$ because the efflux kinetics of 16:0 from BSA-free sheep and human ghosts are essentially monoexponential with the slope β equal to $k_3^*$ of model II ($B >> E$) from which the much smaller $k_3$ can be evaluated only approximately.

**Table 3.** The rate constants ($k_3$) of fatty acid membrane transfer of sheep and human red cells and free activation energy Δ G* of rate constants from human cells unidirectional efflux (column 4) and of netto transfer determined by Kleinfeld et al. [21] (column 5)

| FA | $k_3$ $sec^{-1}$ Sheep cells 0°C | $k_3$ $sec^{-1}$ Human cells 0°C → 37°C$^{\Delta}$ | | Δ G* kcal/mol, 37°C | Δ G* kcal/mol, 37°C |
|---|---|---|---|---|---|
| 20:4 | 0.15 ± 0.02 | 0.39 ± 0.03 | → 1.60 | 18.0 | ND |
| 18:2 | 0.065 | 0.185 | → 0.75 | 18.6 | 17.3 |
| 18:1 | 0.035 | 0.067 | → 0.28 | 19.2 | 18.1 |

$^{\Delta}$: zero degree values of $k_3$ are converted to 37°C values according to the activation enthalpy of 7 kcal/mol [21] neglecting the term RT. ND means not determined.

## Discussion

FA binding and transport by sheep ghost membranes show no phase transition between 0° and 37°C. These findings are in accordance with results obtained with human ghosts. Binding capacities were independent of temperature and the rate constants varied monotonically with temperature. At 37°C binding capacities per unit areas of sheep membranes of four FA are indistinguishable from those of human cells and the same is the case for the affinities of the four FAs (Table 1). Using human values at 0°C in double tracer binding studies with sheep cells, the binding capacities of pairs of FA are partly overlapping, exactly as described for human cells [12]. A temperature independence of the lipids of a plasma cell membrane i.e., no phase transition temperature within a large temperature interval has been described [3].

At near 0°C, tracer exchange efflux of arachidonic acid from ghosts without enclosed BSA was, as expected, biexponential (Fig. 2*A*) and the compartment analysis according to the model I (Fig. 1), gave values of $B/E$ and $k_1$ which were indistinguishable from the values found in studies of human cells. However, the value of the rate constant $k_3$ was much smaller 0.15 ± 0.02 $sec^{-1}$ compared with 0.39 ± 0.03 $sec^{-1}$. As in studies of human cells we ensured that the parameters also determine the FA transfer between FA-BSA pools within and outside the membrane by analysis of the much slower tracer exchange kinetics from cells containing BSA. Also for 18:2 is the tracer efflux in similar experiments with sheep cells slower than with human cells and fitting the $k_3$ value, which provides satisfactory agreement between the measured and the theoretical values showed again that $k_3$ for the sheep cell membrane is much smaller than for the human cell membrane. A similar fitting procedure provides evaluation of $k_3$ for 18:1. In Fig. 4 we show the correlations between $k_3$ values and the number of FA double bonds for sheep and human cells. (These correlations are not compatible with a carrier transfer theory).

In a preceding paper [12] we suggested that the effect of double bonds points to transfer by simple diffusion, because it is reasonable that double bonds could diminish the probability of movement of hydrocarbon chains in sections. Eyring [17] suggested an explanation for an activation energy of long-chain hydrocarbon flow being considerably smaller than the expected fraction of

the heat of evaporation. The fractions decrease with increasing chain length because the long chains commence to move in sections, requiring smaller holes and activation energies. This flexibility is revealed by means of $^2$H-NMR analyses of bilayers and biological membranes containing $^2$H-position labeled FA [25]. The molecular order parameters are constant from the head group until $C_6$ and then decrease towards the methyl terminal due to trans-gauche rotational isomerization. Also the studies reveal stiffness of the oleic acid double bond. Thus such stiffness may account for the smaller $k_3$ in the sheep membrane for all three FA because the cell membranes have no phosphatidylcholine, only sphingomyelin with longer and more saturated hydrocarbon chains [14]. This means a higher viscosity for the movements of FA and thus smaller $k_3$ values for sheep red cells relative to human red cells. A theory on the short range transversal diffusion of FA in the bilayer must be different from that of the fast long range lateral diffusion for which a conventional diffusion coefficient can be defined [1]. However, Eyring [17] defined a diffusion coefficient according to his rate theory as $D = \lambda^2/t$, where λ is the distance between two subsequent equilibrium positions, in general the size of the molecule, and $t$ is the time period between successful jumps. This concept is readily applicable to FA translocation within the bilayer where λ is of the order of FA length (2 nm) and $1/t$ is the frequency of translocation ($k_3$). Accordingly, one can calculate diffusion coefficients of about $10^{-14}$ cm$^2$ sec$^{-1}$, values not far from those reported for bilayers in the gel phase [1]. The reason for this low coefficient is elucidated by the large negative activation entropy calculated from the free activation enthalpy ($\Delta H^*$, for oleic acid about 9 kcal/mol (*unpublished*)) and the free activation energy of the diffusion $\Delta G^*$ which is defined by $k_3 = 1/t = (\kappa T)/h\, e^{-\Delta G^*/RT}$ where the physicochemical constants κ, $T$, and $R$ have conventional meanings. This theory has been applied in a recent study by Kleinfeld et al. [21] on FA transfer flip-flop rates in the human red cell membrane. By directly recording FA efflux from loaded ghost membranes the estimated flip-flop rate constants at 20° and 38°C have been reported. We have extrapolated our 0°C values to 37°C for 18:1 and 18:2 (Table 3). These values are in agreement with the values reported by Kleinfeld et al. [21] and not significantly different, which is significant because the methods used are entirely different. On the other hand, Kleinfeld et al. [21] report that phase partitions between ghost phospholipids and water-phase FA are independent of the load, suggesting no high affinity elements. This is in contrast to Goodman [18] and to our results [12] on which the kinetic analysis is based. We believe that the reported high partition coefficients, independent of the load of FA added directly to suspended ghosts are probably not true phase partitions of monomers since dimerization is a well-known phenomenon for FA added to water [26]. According to our data with BSA buffered FA, high partition values, such as those reported by Kleinfeld et al. [21], arise only with $v \leq 0.2$ and decreases some 10–15-fold for $v \to 3$. Fortunately, prevalent monomers were ensured by BSA buffering in measurements of flip-flop rates and the measured water phase concentrations were within the range, saturating the high-affinity binding elements according to our studies [12], $v \leq 3$. Thus the same membrane elements transfer rates of FA have been measured as in our work.

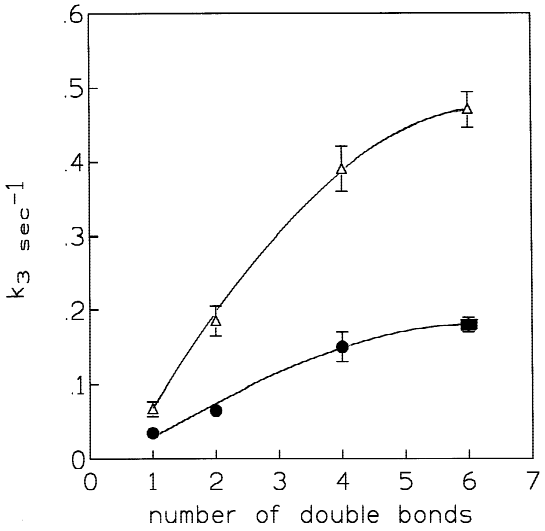


**Fig. 4.** The rate constant ($k_3$) of unidirectional flow through the ghost membrane from the inner surface FA pool (*B*) to the outer surface FA pool (*E*) dependency of the number of double bonds in FA. ● sheep ghost membranes, Δ human ghost membranes. The FAs studied are oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4) and docosahexaenoic acid (22:6). ■ most recently we have determined the membrane transfer rate constant in sheep cells for docosahexaenoic acid (22:6) to 0.18 ± 0.01 sec$^{-1}$.

The elements with high affinities for unsaturated FA present the same fraction of surface areas in the smaller sheep cells as in the human cells (Table 1). This suggests a constitutive property and that the domains may be defined by one or more proteins. The anionic monomers have almost the same affinity to the domains as to BSA, which binds the hydrophilic anion groups electrostatically [5]. We have therefore suggested [12] that a similar electrostatic binding occurs in the domains, possibly to positively charged protein groups, catalyzing protonation, whereas the hydrocarbon tails are bound by adapting annular lipids, with a relatively slow exchange [22]. Experiments with phospholipid vesicles containing a pH indicator (pyrenin) have shown a very rapid but stable acidification, when mixed with buffers containing BSA-FA complexes, $v = 2$ or 4 [20]. This phenomenon can be assigned to a vesicle permeation of only un-ionized FA. Thus the FA binding by the domains must involve

protonation, but so far we have been unable to support the hypothesis that positive charges of proteins are required for electrostatic binding and protonation of the anionic FA (*unpublished*). However, proteins may determine the number of transporting elements via the configurations of annular lipids, different from the average [3, 24]. This possibility perhaps is increased by recent DNA studies [19] suggesting that a diversity of proteins with common motifs from unrelated organisms enhance membrane transfer of FA. The red cell membrane transport capacity is representative of many cell types, because the maximum transfer rate of 18:1 at 37°C ($k_3 \cdot B_{max}$) of human ghosts per $\mu m^2$ surface area ($4.4 \times 10^{-10}$ nmoles/min) is comparable with $V_{max}$ for cardiomyocytes ($1.4 \times 10^{-10}$ or $7.6 \times 10^{-10}$ nmoles/min/$\mu m^2$, different reports [15, 28]). When comparing the transport capacities of different cells, it should be recalled that the $k_3$s of sheep red cells are less than half that of human cells although the binding capacities per surface area are identical.

## Conclusion

The surface area of a sheep red cell is somewhat less than that of a human cell and binding capacities of four FA are correspondingly smaller, whereas the affinities are similar. As with human cells, sheep cells show similar competitive binding of unsaturated FA to the very different and partly overlapping capacities, some of which probably are limited by the available lipids.

As with human cells, the unidirectional rate constants of FA flow between the two membrane leaflets of sheep red cell are correlated to the number of double bonds, but they are less than half the rate constant found for the human red cell membrane. This finding is reasonable because sheep membranes contain sphingomyelins with very long chain saturated FAs instead of phosphatidylcholine rich in unsaturated FA. The results suggest transfer by diffusion of the lipophilic neutral form of FA produced when the FA-anions are bound in specific annual domains.

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## References

1. Almeida, P.F.F., Vaz, W.L.C. 1995. Lateral diffusion in membranes. *In:* Handbook of Biological Physics. Vol. 1. R. Lipowsky and E. Sackmann, editors. pp. 305–357. Elsevier Science B.V. North-Holland
2. Berk, P.D. 1996. How do long-chain free fatty acids cross cell membranes? *Proc. Soc. Exp. Biol. Med.* **212:**1–4
3. Bloom, M., Mouritsen, O.G. 1995. The evolution of membranes. *In:* Handbook of Biological Physics. Vol. 1. R. Lipowsky and E. Sackmann, editors. pp. 65–95. Elsevier Science B.V. North-Holland
4. Bojesen, E., Bojesen, I.N. 1986. Evaluation of chemical parameters of four systems of radioimmunological assay. *Scand. J. Clin. Lab. Invest.* **46:**1–17
5. Bojesen, E., Bojesen, I.N. 1996. Albumin binding of long-chain fatty acids: Thermodynamics and kinetics. *J. Phys. Chem.* **100:**17981–17985
6. Bojesen, I.N., Bojesen, E. 1990. Fatty acid-binding to erythrocyte ghost membranes and transmembrane movement. *Mol. Cell. Biochem.* **98:**209–215
7. Bojesen, I., Bojesen, E. 1991. Palmitate binding to and efflux kinetics from human erythrocyte ghost. *Biochim. Biophys. Acta* **1064:**297–307
8. Bojesen, I., Bojesen, E. 1994. Different arachidonate and palmitate binding capacities of the human red cell membrane. *J. Membrane Biol.* **142:**113–116
9. Bojesen, I.N., Bojesen, E. 1995. Arachidonic acid transfer across the human red cell membrane by a specific transport system. *Acta Physiol. Scand.* **154:**253–267
10. Bojesen, I.N., Bojesen, E. 1996. Oleic acid binding and transport capacity of human red cell membrane. *Acta Physiol. Scand.* **156:**501–516
11. Bojesen, I.N., Bojesen, E. 1996. Specificities of red cell membrane sites transporting three long chain fatty acids. *J. Membrane Biol.* **149:**257–267
12. Bojesen, I.N., Bojesen, E. 1998. Nature of the elements transporting long-chain fatty acids through the red cell membrane. *J. Membrane Biol.* **163:**169–181
13. Deenen van L.L.M., deGier, J. 1964. Chemical composition and metabolism of lipids in red cells of various animal species. *In:* The Red Blood Cell. C. Bishop and D.M. Surgenor, editors. pp. 279. Academic Press, New York and London
14. Deuticke, B. 1977. Properties and structural basis of simple diffusion pathway in the erythrocyte membrane. *Rev. Physiol. Biochem. Pharmacol.* **78:**1–97
15. Diede, H.E., Rodilla-Sala, E., Gunawan, J., Manns, M., Stremmel, W. 1992. Identification and characterization of a monoclonal antibody to the membrane fatty acid binding protein. *Biochim. Biophys. Acta* **1125:**13–20
16. Frézard, F., Garnier-Suillerot, A. 1998. Permeability of lipid bilayer to anthracycline derivatives. Role of the bilayer composition and of the temperature. *Biochim. Biophys. Acta* **1389:**13–22
17. Glasstone, S., Laidler, K.J., Eyring, H. 1941. The Theory of Rate Processes. McGraw-Hill Book, New York
18. Goodman, De Witt, S. 1958. The interaction of human erythrocytes with sodium palmitate. *J. Clin. Invest.* **37:**1729–1735
19. Hirsch, D., Stahl, A., Lodish, H.F. 1998. A family of fatty acid transporters conserved from mycobacterium to man. *Proc. Natl. Acad. Sci. USA* **95:**8625–8629
20. Kamp, F., Hamilton, J.A. 1992. pH gradients across phospholipid membranes caused by fast flip-flop of un-ionized fatty acids. *Proc. Natl. Acad. Sci. USA* **89:**11367–11370
21. Kleinfeld, A.M., Storms, S., Watts, M. 1998. Transport of long chain native fatty acids across human erythrocyte ghost membranes. *Biochemistry* **37:**8011–8019
22. Marsh, D. 1985. Esr spin label studies of lipid-protein interaction. *In:* Progress in Protein-Lipid Interactions. Watt and DePont, editors, pp. 143–172. Elsevier Sc. Publ. B.V. Amsterdam, New York

23. Noy, N., Donelly, T.M., Zakim, D. 1986. Physical-chemical model for the entry of water-insoluble compounds into cells. Studies of fatty uptake by the liver. *Biochemistry* **25:**2013–2021
24. Sackmann, E. 1995. Physical basis of self-organization and function of membranes: Physics of vesicles. *In:* Handbook of Biological Physics. Vol. 1. R. Lipowsky and E. Sackmann. editors. pp. 213–304. Elsevier Science B.V. North-Holland
25. Seelig, J., Seelig, A. 1980. Lipid conformation in model membranes and biological membranes. *Quart. Rev. Biophys.* **13:**19–61
26. Smith, R., Tanford, C. 1973. Hydrophobicity of long chain n-alkyl carboxylic acids as measured by their distribution between heptane and aqueous solutions. *Proc. Natl. Acad. Sci. USA* **70:**289–293
27. Sorrentino, D., Stump, D., Potter, B.J., Robinson, R.B., White, R., Kiang, C.-L., Berk, P.D. 1988. Oleate uptake by cardiac myocytes is carrier mediated and involves a 40-kD plasma membrane fatty acid binding protein similar to that in liver, adipose tissue, and gut. *J. Clin. Invest.* **82:**928–935
28. Stremmel, W. 1989. Transmembrane transport of fatty acids in the heart. *Mol. Cell. Biochem.* **88:**23–29
29. Zakim, D. 1996. Fatty acids enter cells by simple diffusion. *Proc. Soc. Exp. Biol. Med.* **212:**5–14