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Nature of the Elements Transporting Long-Chain Fatty Acids Through the Red Cell Membrane

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Abstract. Docosahexaenoic acid is found to be bound to three equivalent sites on albumin with the same affinities as palmitic acid at 0–38°C, which demonstrates that ethene-1,2-diyl- and methylene-groups contribute equally to the affinity. The equilibrium dissociation constants (K_{dm}s) for red cell membrane binding sites of linoleic- and docosahexaenoic acid at pH 7.3 are determined at temperatures between 0 and 37°C. The temperature-independent capacities for binding are 12 ± 1 and 25.4 ± 3.0 nmoles g^{-1} ghosts respectively. Double isotope binding experiments reveal that the unsaturated fatty acids: arachidonic-, linoleic-, docosahexaenoic-, and oleic acid have partially shared capacities in ratios approximately 1:2:4:5, in contrast to the noncompetitive binding of palmitic acid. The observations suggest a two-tier binding limitation. One is the number of protein sites binding fatty acid anions electrostatically and the other is the number of suitable annular lipids adaptively selected among membrane lipids by the hydrocarbon chain. These competition conditions are confirmed by measurements of the tracer exchange efflux at near 0°C from albumin-free and albumin-filled ghosts of linoleic- and docosahexaenoic acid, either alone or in the presence of arachidonic- and palmitic acid. Under equilibrium conditions, the calculated ratios of inside to outside membrane binding is below 0.5 for four unsaturated fatty acids. The unidirectional rate constants of translocation between the inside and the outside correlate with the number of double bonds in these fatty acids, which are also correlated with the dissociation rate constants of the complexes with albumin. The membrane permeation occurs presumably by binding of the anionic unsaturated fatty acids to an integral protein followed by channeling of the neutral form between opposite binding sites of the protein through annular lipids encircling the protein.

Key words: Docosahexaenoic acid (Δ 4,7,10,13,16,19) — Linoleic acid — Red cell membrane — Transporting elements — Transport kinetics — Fatty acid transport

Introduction

Plasma membrane binding and transport of long chain fatty acids (FAs) are important and highly controversial problems [6, 39]. Transport by carriers is suggested by kinetic studies on the uptake and release of FAs by metabolizing cells [*see* Table 1 [6], 21] and subsequently the proteins that are the presumptive carriers have been isolated. Different experimental procedures suggest four different candidates for only one kind of cell, adipocytes [*see* Table 1 [6]], with no specificity for a particular FA. In three studies, the observation of fairly specific inhibitions of presumptive carrier systems strongly supports the carrier theory but the inhibitions are incomplete, indicating that one or several different carriers may be present. The carrier theory has been refuted by other researchers on the basis of transport studies with bilayers of liposomes, which by the authors were considered to be a satisfactory model for the lipid bilayers in plasma membranes [20, 29, 32].

In fact, the transport process of FA across ghost membranes is a venerable problem. The early work of Goodman [24] showed that FA monomer binding by red cell membranes is saturable and orders of magnitude greater than expected from equilibrium partition between hydrocarbon and water phases. This work suggested for the first time the presence of an FA "receptor" in a plasma membrane and is in accordance with the results of later works, which suggest an FA carrier and leave

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little room for a phase partition dependent binding and translocation.

Our works on FA binding and transport by the resealed red cell membrane, ghosts, [10, 11, 14, 15, 16, 17] and on FA bindings to bovine serum albumin (BSA) extend the work of Goodman [24]. The present work on unsaturated FAs is an attempt to elucidate the nature of the "receptor" for which we prefer the term "elements" because the structure in question probably includes the annular lipids of one or more integral proteins.

Recently we showed [17] that ghost bindings of 16:0 and of two unsaturated FAs, 20:4 and 18:1, are independent, in contrast to the albumin bindings for which all the FAs have three equivalent binding sites [7]. By extending our studies to 18:2 and 22:6 we have found very different binding capacities and transport kinetics for two different unsaturated FAs. They share however more or less membrane bindings competitively, suggesting a two-tier binding to a protein and its rapidly exchanging annular lipids as discussed. These observations together with transport data suggest a membrane transport model that may corroborate data on presumptive carrier transport of FA and some information on lipid bilayer permeation.

Materials and Methods

Materials

[4,7,10,13,16,19-[4,5-^{3}H]] Docosahexaenoic acid, sp. act. 58 Ci/mmol and [^{14}C(U)] linoleic acid, sp. act. 1.045 Ci/mmol were purchased from DuPont NEN, Boston, MA. Unlabeled fatty acids (FAs) are from Sigma. Labeled as well as unlabeled FAs were purified monthly by column chromatography, ensuring elution of a single component. The scintillation fluid Ultima Gold was purchased from Packard Instrument Company, (Downers Grove, IL) Bovine serum albumin (BSA) (Behring Institute, Germany) was defatted as previously.

Experimental and Theoretical Methods

Preparation of Ghosts and Buffer with FA-BSA Complexes

BSA-filled ghosts and BSA-free ghosts were prepared by hypotonic hemolysis at 0°C in media with or without BSA [11]. After resealing of ghosts at 38°C for 45 min, the pink ghosts were washed thrice with 165 mM KCl, 2 mM phosphate buffer, pH 7.3 containing 0.02 mM EDTA/EGTA (1:1) and once with the same buffer containing BSA, in appropriate concentrations or corresponding to intracellular concentrations and stored herein.

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 (v) from 0.25 to 2.25 was deposited on glassbeads as described previously [16]. These were shaken with the buffer containing BSA for 15 min at room temperature.

Labeling of Ghosts and Uptakes of FAs

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 v values, as $C_a/(S_a \times [BSA])$.

Estimations of Water Phase Monomer Concentrations [FA] in Equilibrium with FA-BSA

The method has been described previously [12, 13]. In short, after labeling BSA-filled ghosts and counting of the buffer to get v, as described above, the ghosts were washed 4 times with 50 volumes of buffer without BSA at 4°C. To correct for any tiny amount of binding capacity that may be released from ghosts during this procedure, a series of dilutions is carried out according to the following scheme: Ghosts (0.5 ml) were suspended in buffer to a total volume of 3 ml and four tubes were prepared {i} 1.25 ml ghost suspension to 1.75 ml buffer; {ii} 0.75 ml to 2.25 ml buffer; (iii) 0.5 ml to 2.5 ml buffer and (iv) 0.25 ml to 2.75 ml buffer. The four suspensions were gently shaken in a water bath at the labeling temperature and after complete equilibration, ghosts were sedimented by centrifugation as described above and 2 × 200 μl ghosts-free supernatants were pipetted from the top of each tube for scintillation counting (C_w dpm ml^{-1}) in order to determine [FA] in the water phase (essentially anionic FA concentration) as C_w/S_a. The efficiency for 3H and 14C in unquenched samples were 67 and 90% respectively when counted separately. In dual-label counting it was 56 and 83% respectively.

The equilibrium constant, K_d, for the dissociation of FA from BSA is defined by

$$K_d = ([\mathrm{FA}]\,(N - v))/v \tag{1}$$

Corresponding values of v and [FA] gives estimates of N and K_d according to the linearization of Eq. (1)

$$[\mathrm{FA}]/v = [\mathrm{FA}]/N + K_d/N \tag{2}$$

Fatty Acid Uptake by Ghosts

As described above BSA-free ghosts were equilibrated with the solutions containing radioactive FA. The amount of FA bound to the membrane (M) in nmol g^{-1} ghosts, and final v values are calculated on the basis of counting rates in the solutions before and after equilibration and the specific activities of FA or directly on the basis of weighted samples of packed ghosts after removal of the supernatant and washing, taking into consideration the extracellular volume trapped in packed ghosts. The equilibrium constants, K_{dm}s, for the dissociation of FA from binding site on the ghost are

$$K_{dm} = ([\mathrm{FA}]\,(C - M))/M$$

[FA] is defined by the equilibrium constant, K_d, for dissociation of FA-BSA complex and v of the BSA buffer with which the ghosts are equilibrated. Combining the definitions of K_d and K_{dm} and eliminating [FA] gives corresponding M and v values. With three binding sites on BSA we get:

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

The capacity and the ratio of equilibrium constant can be estimated from such linear regressions.

Calculated Membrane Binding of Two Different FAs

Uptakes are measured in double isotope studies of two FAs bound to albumin in various v values. The monomer concentration of one fatty acid [FA_1] in the presence of another is estimated by:

$$[FA_1] = K_{d1}\, v_1/(3 - (v_1 + v_2)) \tag{4.1}$$

With the analogous Eq. for [FA_2]

$$[FA_2] = K_{d2}\, v_2/(3 - (v_1 + v_2)) \tag{4.2}$$

In the calculations, the FA of highest capacity is called FA_2 i.e., the shared capacity is in all cases C_1. The binding m_1 of FA_1 by ghost elements, capacity C_1, in competition with FA_2 and the binding m_{2o} of FA_2 in competition with FA_1 in C_1 are given 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. These are:

$$m_1 = C_1\, [FA_1]/[K_{dm1}(1 + [FA_2]/K_{dm2}) + [FA_1]] \tag{5.1}$$

and combining Eqs. (5.1, (4.1) and (4.2)

$$m_1 = C_1/[(K_{dm1}/K_{d1})(3\text{-}v_1\text{-}v_2)/v_1 + (K_{dm1}\, K_{d2}\, v_2)/ (K_{d1}\, K_{dm2}\, v_1) + 1] \tag{6}$$

and

$$m_{2o} = C_1\, [FA_2]/[K_{dm2}(1 + [FA_1]/K_{dm1}) + [FA_2]] \tag{5.2}$$

and combining Eqs. (5.2), (4.1) and (4.2)

$$m_{2o} = C_1/[(K_{dm2}/K_{d2})(3\text{-}v_1\text{-}v_2)/v_2 + (K_{dm2}\, K_{d1}\, v_1)/ (K_{d2}\, K_{dm1}\, v_2) + 1] \tag{7}$$

From (6) and (7) m_1 and m_{2o} can be calculated exclusively from estimated parameters.

The membrane still has a residual binding capacity ($C_2 - C_1$) for FA_2. This binding is called m specific (m_{2sp}) and it can be calculated as

$$m_{2sp} = (C_2 - C_1)/(1 + K_{dm2}/[FA_2]) \text{ or}$$
$$m_{2sp} = (C_2 - C_1)/(1 + (K_{dm2}/K_{d2})(3\text{-}v_1\text{-}v_2)/v_2) \tag{8}$$

using [FA_2] given by Eq. (4.2).

$$\text{In Table 3 } m_2 = m_{2o} + m_{2sp} \tag{9}$$

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 glassbead technique (*see above*), but up to 2–3 g of glassbeads were used for disposition of FA.

Serial sampling of cell-free extracellular medium was done as described previously [11] and the radioactivity of 400 μl samples was measured by scintillation counting.

Figure 1 shows the compartment models we have used to account for the tracer efflux kinetics from ghosts. The mathematics of the corresponding equations and expression have been published in detail previously [14]. Here we only give the equations used to estimate the model parameters from the integration constants and the rate coefficients. The biexponential efflux kinetics predicted by the model I (three compartments) has the solution:

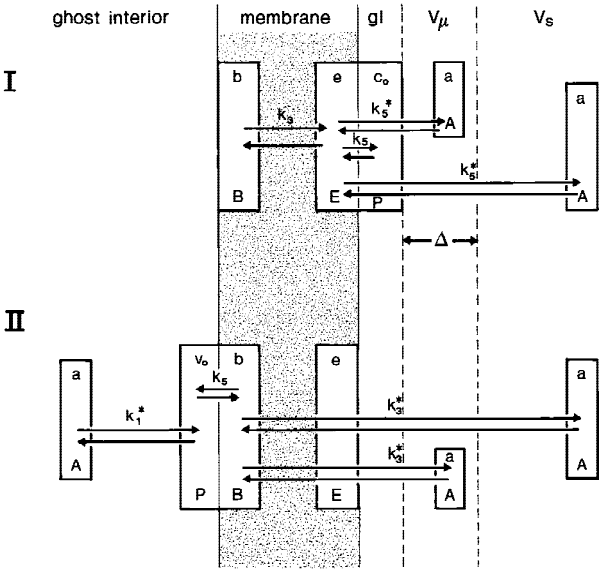

Fig. 1. The compartment models used to account for efflux of tracer-fatty acid from ghosts in nonisotopic equilibrium with the medium. I: Ghosts without serum albumin. II: Ghosts with serum albumin. Arrows indicate unidirectional fatty acid fluxes. k_1^*, k_3, k_3^*, k_5 and k_5^* are first order rate constants of fluxes between adjacent compartments. Lower case letters a,b,e,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 called [FA]. V_u is unstirred volume around ghosts with the depth Δ and V_s the stirred volume ghosts. gl is a 10-nm deep glycocalyx space. Reprinted with permission from *Acta Physiologica Sandinavica* (1995) **154:**253–267.

$$(1 - y/y_\infty) = K_1\, e^{-\alpha t} + K_2\, e^{-\beta t} \tag{10}$$

and the parameters B/E, k_3 and k_5^* are derived from the following equations:

$$k_5^* = \alpha + \beta - \alpha\,\beta/(\alpha K_1 + \beta K_2) \tag{10a}$$
$$k_3 = \alpha\,\beta/k_5^* \tag{10b}$$
$$1 + B/E = k_5^*/(\alpha K_1 + \beta K_2) \tag{10c}$$

and k_1 by

$$k_1 = \left[\frac{k_5^* C\,(3-\nu)\,10^{-6}}{(1 + B/E)\,(K_{dm}\,(3-\nu) + K_d\nu)} - 1/R_D}{S10^{-12}\sqrt{D}}\right]^2 \left(\frac{K_d}{(3-\nu)\,[BSA_y]}\right) \tag{10d}$$

For k_5^*, k_3, B and E *see* Fig. 1 and Appendix. [BSA_y] is the BSA concentration in the efflux medium.

The biexponential efflux kinetics predicted by model II (a three-compartment approximation) has the solution:

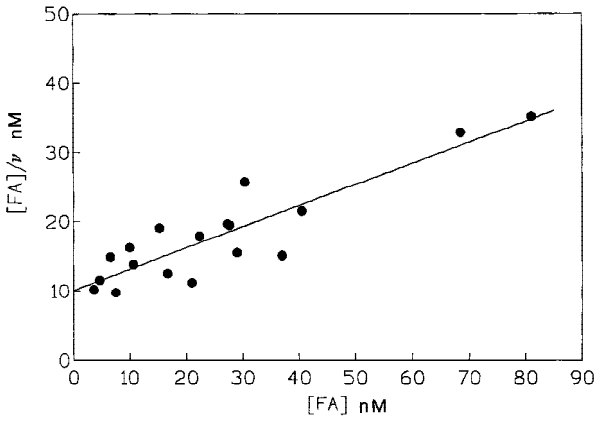

Fig. 2. Relationship between docosahexaenoic acid (22:6) monomer water-phase concentrations ([FA] nM) and the molar ratio of FA to BSA (ν) presented according to the linearized definition of the equilibrium constant K_d of N equivalent binding sites (Eq. (2) in the method section) at 38°C. The regression line is [FA]/ν = 0.306 (±0.038) [FA] + 10.11 (±1.26), r = 0.92.

$$(1 - y/y_\infty) = K_3\, e^{-\gamma t} + K_4\, e^{-\delta t} + f_e\, e^{-k_5 \cdot t} \qquad (11)$$

$K_3 + K_4 + f_e = 1$, f_e being the tracer fraction initially present in the E compartment.

From this equation we can get an experimental $\delta^* = \delta/(1 + \delta/\gamma)$. The integration constants K_3 and K_4 and the rate coefficients γ and δ are related to the theoretical constants k_3^*, k_5 and k_1^* *(see* Fig. 1) by the following equations:

$$\gamma\delta = k_3^* k_5 Q/(1 + Qk_5/k_1^*)$$

$$\gamma + \delta = (k_3^* + k_5(1 + Q) + k_3^*\, k_5\, Q/k_1^*)/(1 + Qk_5/k_1^*)$$

Q is the ratio of FA in transport pool B to FA bound to BSA within ghosts and k_1^* is the virtual dissociation rate constant of BSA-FA complex in the unstirred cellular volume [15].

From these two equations we get a corresponding theoretical δ^* (theor)(*see* Table 6)

$$\delta^*\,(\text{theor}) = \frac{Q}{1/k_5 + 1/k_3^* + Q\,(1/k_3^* + 1/k_1^*)} \qquad (12)$$

Here $1/k_5$ is neglected compared to $1/k_3^*$ as $k_5 \gg k_5^*$.

Statistics and Data Analyses

We have used the standard methods [4] to calculate the statistics, weighted means, probabilities of significance of differences and of correlation coefficients. The SEs of complex expressions are computed from SEs of the components by numeric differentiation [8] neglecting covariations. The nonlinear regression procedure given by STATGRAPHICS version 5 was used to determine the best fit of the data to a sum of two exponentials with known distributions.

Results

Albumin Binding of Long-Chain Fatty Acids

It is a prerequisite of the present study that the monomer concentrations of FA are well-defined. This requires buffering with FA bound to albumin. Previous studies have shown that BSA has three equivalent high affinity sites for 16:0, 18:1, 18:2 and 20:4 at temperatures between 0–38°, when ν is kept within physiological values [9]. Experiments with 22:6 demonstrate that the number of equivalent high affinity sites (N) is not different from three at different temperatures from 0 to 38°C. Figure 2 shows an example of data analyses after linearization of the relation between the two parameters (ν and [FA]) according to Eq. (2). The plot at 38°C gives $N = 3.27 \pm 0.41$ and $K_d = 33.0 \pm 5.8$ nM. In the following analyses we use the K_d values for the five FAs and calculated for $n = 3$, shown in Table 1 for various temperatures.

Ghost Bindings of FAs

In previous studies [17] we showed that ghosts have a limited capacity for the binding of 16:0, 18:1 and 20:4 and that the equilibrium constants are defined by K_{dm} = [FA] (C-M)/M (*see* Methods). Previous studies on 16:0, 20:4 and 18:1 revealed that plots according to Eq. (3) are linear for ν <2.5. This demonstrates a constant C and that there is only one equilibrium constant (K_{dm}). Regression analyses of data on 18:2 and 22:6 at 37°C according to Eq. (3) are shown in Fig. 3. Such plots have been used to determine values of C and K_{dm}/K_d at various temperatures. Table 2 presents the values of C, K_{dm}/K_d and K_{dm} (calculated from known values of K_d) for all five FAs. C (in nmoles g^{-1}) ranges from about 6 for 20:4 to 34 for 18:1. As expected C is essentially temperature independent and different for different FAs. Comparison (Table 1 and 2) shows that the equilibrium constants, K_{dm} and K_d, are of similar magnitudes, but whereas the affinities to BSA depend considerably on the temperature, this is not the case for the membrane bindings of unsaturated FA, except a little for 22:6.

The Membrane Bindings of Unsaturated FA are Partly Competitive

The equilibrium uptake by ghosts has been studied in double isotope experiments with two different FAs labeled with either ^{3}H or ^{14}C. Different values of ν for the two FAs are used in different experiments, but the sum is smaller than 2.5. In contrast to the noncompetitive bindings of 16:0 and two unsaturated FAs (20:4 and 18:1 [17]) it is immediately clear, that two unsaturated FAs mutually inhibit the binding of each other (Fig. 4). The binding capacities of ghost membranes for FAs are very different and thus an FA for which the capacity is low may diminish only a part of the binding of another FA for which the capacity is higher. The uptakes (M_1 and M_2)

of four pairs of unsaturated FAs are compared with predicted uptakes (m_1 and m_2) calculated according to Eqs. (6), (7), (8) and (9) (Table 3). In the calculation no cooperative effects have been included. We have assigned the binding capacity C_1 (for FA_1) as the lowest capacity and the larger capacity for FA_2 as C_2. FA_2 and FA_1 share the capacity C_1 in the ratio indicated by R, but the membrane still has a residual capacity (C_2-C_1) for binding of FA_2 alone. The validity of the presumptions are confirmed by the generally insignificant differences between measured and calculated values, except for two cases for which statistical analysis gives (M_1-m_1) and (M_2=m_2) with $P < 0.02$. The results of representative experiments for the four groups are shown in Fig. 4. The mutual inhibitions appear when uptakes with no competition between two FAs bound to BSA (FA_1 and FA_2) which are indicated by the sizes of the columns are compared with the actual uptakes which are indicated by the grey areas. Thus in the case of the pair III, the uptake of FA_1 (20:4) is only slightly depressed by the presence of FA_2 (22:6), whereas FA_2 uptake is greatly depressed by the presence of FA_1. This is explained by the much smaller K_{dm}/K_d ratio for 20:4 than for 22:6 which implies only a small uptake of 22:6 by the shared bindings sites (dark grey area) besides the specific uptake in (C_2-C_1) (light grey area).

According to Table 3, 22:6 and 18:2 compete with 20:4 for the same capacity, defined by the 20:4 binding alone, and since 18:1 competes with binding of 22:6 we conclude that 18:1 is bound by the same membrane sites. A second type of site binds 18:2, 22:6 and 18:1 but not 20:4. A third kind binds 22:6 and 18:1 whereas a fourth kind only binds 18:1 among the 4 unsaturated FAs studied. Table 4 shows that 16:0 is not interferring with the binding of 22:6. Since the largest 18:1 binding capacity is shared differently by the other three unsaturated FAs the number of binding sites of the four unsaturated FAs are approximately the same as that of 16:0.

Exchange Efflux of 18:2 and 22:6 from Ghosts without BSA to BSA in Media

These experiments quantify the FA transport properties of the binding sites. In Fig. 1 is shown the two compartment models we have used to account for the efflux kinetics of FA from ghosts. Equations (10*a*–10*d*) presented in Materials and Methods are used to convert the constants of the biexponential efflux curves to the parameters of the model. Thus, we calculate the three parameters: (i) the distribution of bindings to the inner and outer membrane compartments, *B/E* (*see* Fig. 1) where $(B + E)_{max} = C$, (ii) the $B \rightarrow E$ unidirectional rate constants, k_3, and (iii) the dissociation rate constants of the FA-BSA complex, k_1. The efflux is very fast, however,

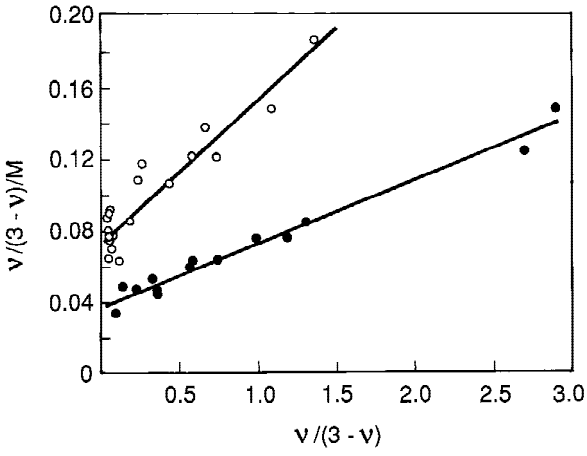

Fig. 3. Regression analysis of red cell membrane binding at 37°C of docosahexaenoic acid (22:6): ● and of linoleic acid (18:2): ○ according to the Eq. (3) in methods. $(\nu/(3-\nu))/M = (\nu/(3-\nu))\ 1/C + (K_{dm}/K_d)\ 1/C$ where ν is the molar ratio of fatty acid to albumin, M the membrane binding of fatty acid and C the binding capacity of ghosts both in nmoles fatty acid per g ghosts, K_{dm} is the equilibrium constant for fatty acid dissociation from ghost membranes and K_d the equilibrium constants for fatty acid dissociation from albumin with three equivalent sites.
The regression line for (22:6) is $Y = 0.0353(\pm 0.0016)\ X + 0.0377(\pm 0.0020)$, $r = 0.988$. The regression line for (18:2) is $Y = 0.0790(\pm 0.0073)\ X + 0.0732(\pm 0.0033)$, $r = 0.93$. The regressions provide estimates of the slope ($1/C$) and the intercept ($(K_{dm}/K_d)\ 1/C$).

the resolution time of about 1 sec at zero degree is small enough to show the expected biexponential kinetics for 18:2 and 22:6, as previously found for 20:4 and 18:1 [15, 16]. Figure 5*A* shows that the exchange efflux of 22:6 is faster than that of 18:2. Figure 5 *B* shows that the efflux rate increases with the BSA concentration of the medium as expected from unstirred layer effect. Table 5 shows the transfer parameters of the five FAs. The binding distribution *B/E* is <0.5 for all the unsaturated FAs in contrast to about 4 for 16:0. The two rate constants, k_3 and k_1, are both significantly correlated with the number of double bonds, as is the unidirectional rate constant $E \rightarrow B$ defined by ($k_3 \cdot B/E$). A statistical analysis with 2 degrees of freedom shows that the correlations are significant ($P < 0.02$) in all three cases. The significance of these results is discussed below.

Exchange Efflux of 18:2 and 22:6 from Ghosts Containing BSA

Efflux experiments were carried out with ghosts containing different intracellular [BSA] at different ν values (Table 6). Data for two representative experiments are shown in Fig 6. *A* and *B*. Final slopes of corresponding semilogarithmic plots (δ) have been calculated. In Ma-

Table 1. Equilibrium constants (K_d, nM) of fatty acid dissociation from BSA calculated for three equivalent sites at different temperatures

Fatty acid	Temperatures				
	0°C	10°C	23°C	38°C	
16:0	4.10 ± 0.30	8.30 ± 0.80	16.00 ± 1.00	34.00 ± 2.20	$\nu \leqslant 1.4$
18:1	1.21 ± 0.07	2.30 ± 0.20	2.90 ± 0.14	6.60 ± 0.40	$\nu \leqslant 1.8$
18:2	3.50 ± 0.49	5.90 ± 0.30	11.30 ± 1.00	17.00 ± 0.44	$\nu \leqslant 2.3$
20:4	4.90 ± 0.30	7.40 ± 0.40	15.60 ± 0.70	28.00 ± 0.80	$\nu \leqslant 1.5$
22:6	4.10 ± 0.86	13.0 ± 2.5	18.40 ± 2.60	29.00 ± 2.30	$\nu \leqslant 2.5$

Palmitic acid (16:0), Oleic acid (18:1), Linoleic acid (18:2), Arachidonic acid (20:4) and Docosahexaenoic acid (22:6).

Table 2. Parameters of long-chain fatty acid bindings by red cell membranes

Fatty acids	C nmoles · g^{-1}	K_{dm}/K_d			K_{dm} nM		
		0°C	23°C	37°C	0°C	23°C	37°C
16:0	29 (2.1)	0.84 (.17)	0.68 (.06)	0.38 (.06)	3.44 (.74)	10.88 (1.15)	12.9 (2.2)
18:1	34 (2.2)	5.80 (.30)	2.70*	1.38 (.15)	7.02 (.54)	7.83 (.60)	9.11 (.96)
18:2	12 (1.0)	2.95 (.40)	1.16 (.15)	0.92 (.10)	10.3 (2.2)	13.1 (2.0)	15.6 (1.7)
20:4	6 (1.0)	1.03 (.17)	0.36 (.03)	0.17 (.05)	5.05 (.82)	5.62 (.53)	4.8 (1.4)
22:6	27 (4.0)	3.44 (.48)	1.13 (.06)	1.07 (.08)	14.1 (3.5)	20.8 (2.6)	31.0 (3.4)

Membrane binding capacity is C. The ratio of equilibrium constants of fatty acids dissociation from red cell membrane and albumin respectively is K_{dm}/K_d. Numbers in parentheses are SE For abbreviations of fatty acids *see* Table 1. * indicates K_{dm}/K_d calculated from K_d determined at 23°C and a temperature-independent K_{dm} [16].

terials and Methods, we describe the experimental procedure and the equations by which the coefficients δ*(theor) of the rate of efflux can be calculated from Q and the rate constants k_1^*, k_3^*. The latter constants can be calculated from the efflux kinetics from BSA-free ghosts (Eqs. A(12) and A(14) in [16]). In Table 6 we compare the experimental $\delta^* = \delta/(1 + \delta/\gamma)$ with the theoretical δ*(theor) from Eq.(12). In the lower part of Table 6 (III) δ*(theor) is calculated under the assumption that the binding of 22:6 and 16:0 is noncompetitive. In Fig. 6 *B* is shown the efflux of 18:2 in the presence of the competing 20:4 and the noncompeting 16:0 both added to give similar ν values. All three FA compete for BSA binding i.e., the water-phase concentration of 18:2 increases equally in the presence of 20:4 and 16:0. The expected increase in the transport rate of 18:2 is apparent only in the presence of 16:0, whereas the two efflux curves of 18:2 with and without 20:4 are indistinguishable (Fig. 6*A* and *B*). This is readily explained by an inhibition by 20:4 of the transport capacity for 18:2.

Discussion

The Free Energy of Anionic and Neutral FA Transfer from Water to Apolar Structures

Our studies on BSA bindings of monomer FA provide information that is used to account for uptakes of anionic and neutral FA into apolar structures and phases. The present finding that K_d for 22:6 is not different from that of 16:0 (Table 1) confirms that ethene-1,2-diyl- and methylene-groups are equivalent with regard to the free energy of transfer of the anionic FA from water to BSA [9]. Our observation of three equivalent high affinity binding sites on BSA is in agreement with the results of NMR studies on binding between BSA and ^{13}C-carboxyl-FA [25] and with deduction from the three-dimensional model of BSA [18]. It is also in agreement with the dependency of the thermodynamic functions for binding on the structure of the FAs [9]. The BSA bind-

ing sites have a funnel-like structure with cups and spouts [18]. They all bind FA by a combination of electrostatic binding of the anion at positively charged cups and van d. Waal binding of the hydrocarbon tail by the apolar groups of the spouts. The free energies for transfer of different FAs from water to BSA and of FA (minus the -CH_2COOH group) to liquid hydrocarbon [34, 38] are very similar and equal to those obtained by summation of the partial values of methyl, methylene (except for α-CH_2) and ethene-1,2-diyl groups [1]. This strongly suggests that the free energy of a hydrocarbon tail transfer from water to any apolar phase, liquid hydrocarbon, apolar protein domains or membranes can be evaluated in this way. Regarding FA transfer this value can then be added to the contribution from the end group, anionic or neutral. The enthalpic fraction of the free energy for methylene transfer from water to liquid hydrocarbons decreases with the molecular weight of the hydrocarbon [1], which then determines the entropy/enthalpy compensation of "the hydrophobic effect." This phenomenon is important in the case of BSA bindings of FA [9] and applicable to the transfer from water to ghost bindings of unsaturated FAs which primarily is determined by the entropic contribution to the free energy of transfer.

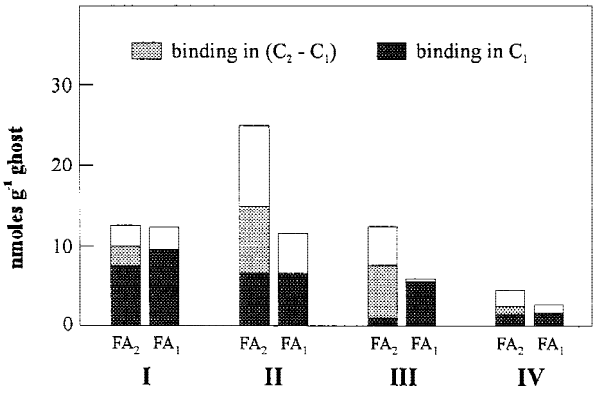

Fig. 4. Partially competitive bindings of pairs of unsaturable fatty acids (FA_1 and FA_2). Representative pairs of the four groups in Table 3. Heights of columns represent estimated membrane bindings of each of the two fatty acids without competition in nmoles g^{-1} ghost. Light grey area signifies the specific binding of FA_2 in membrane capacity (C_2-C_1) according to Eq. (8). Dark grey areas indicate bindings in the shared capacity C_1 (for FA_1). *IV* is carried out at 0°C, the others at 37°C.
I: FA_1 (22:6), $\nu = 0.93$, FA_2 (18:1), $\nu = 0.94$;
II: FA_1 (18:2), $\nu = 1.3$, FA_2 (22:6), $\nu = 1.5$;
III: FA_1 (20:4), $\nu = 1.04$, FA_2 (22:6), $\nu = 0.89$;
IV: FA_1 (20:4), $\nu = 0.80$, FA_2 (18:2), $\nu = 1.4$

Membrane Binding Elements

The uptakes of five different FAs by ghost membranes are saturable (Table 2, Fig. 3) within the FA concentration defined by the high affinity range of the binding to BSA (ν <2.5) as suggested by Goodman in 1958 [24]. We term these saturable binding sites membrane elements. These limited numbers of binding elements preclude any significant contribution from phase partition to the uptake of FAs. On the basis of our data, the amount of lipid in 1 g ghost [2] and Tanford's ΔG° values for partition between aqueous and hydrocarbon phases, it can be calculated that the partition of neutral FAs between water and bilayer lipids is three orders of magnitude lower than the uptake capacity of the binding elements at FA concentrations corresponding to $\nu = 1.5$. The important finding that K_{dm}s and K_ds for the five FAs differ only by small factors (Tables 1 and 2) shows that free energies for transfer of FA anions from water to the membrane elements and to BSA are quite similar. That is, as explained above, the case for the hydrocarbon chains taken alone and must then also be the case for the end group. However, no data are yet available which demonstrate anionic binding directly to constituents of the membrane such as has been shown to BSA [25]. Such electrostatic binding sites for FA anions are probably located in the proteins since the other constituents (phospholipids and glycolipids) are either negatively charged or uncharged. Results of studies by electron spin resonance are indeed in agreement with electrostatic binding of anionic amphipaths by some membrane proteins [31]. Previously, we found that 16:0 and 18:1 or 20:4 binding elements are entirely independent [17]. The present study shows that elements which bind 16:0 do not bind 22:6 (Table 4). In comparison with the unsaturated FAs, 16:0 is exceptional in that the binding has different properties (Tables 2 and 5). Thus K_{dm} of 16:0 increases considerably with temperature and the equilibrium distribution, the *B/E* ratio, is about 4–5 in contrast to *B/E* <0.5 for unsaturated FAs. On the other hand, results summarized in Table 3 and Fig. 4 demonstrate the mutually binding of all the unsaturated FAs. This phenomenon, corroborated by the efflux data (Fig. 6), is intriguing. Different binding capacities preclude application of the concept of specific sites and how it is possible that only one fourth of all the binding elements for 22:6 are available for 20:4 as well. A possible explanation is given below.

The Nature of the Membrane Binding of Unsaturated FAs

The nature of membrane binding may be understood by considering FA bindings to BSA i.e., competitive binding of the carboxyl-group, which is common to all FAs and bindings of the hydrocarbon chains for which any element has specified possibilities, in contrast to the situation in BSA where all the different hydrocarbon chains can be accommodated at the same sites. In BSA, several adjacent coils form the funnel, the spout of which acco-

Table 3. Partially competitive bindings (m and M) of unsaturated long-chain fatty acids by red cell membrane

ν_2/ν_1	R	M_2 nmoles/g	m_2 nmoles/g	M_1 nmoles/g	m_1 nmoles/g
I: FA_1 = 22:6, FA_2 = 18:1					
0.64/0.65	0.76 (0.09)	7.4 (0.3)	6.0 (0.7)	7.13 (0.20)*	6.14 (0.40)
0.93/0.94	0.77 (0.10)	10.2 (0.3)	9.2 (0.8)	9.6 (0.3)	9.13 (0.50)
0.64/0.60	0.83 (0.10)	6.3 (0.2)	5.92 (0.17)	5.7 (0.25)	5.64 (0.33)
0.53/0.46	0.90 (0.10)	4.7 (0.25)	4.8 (0.6)	4.2 (0.2)	4.26 (0.26)
II: FA_1 = 18:2, FA_2 = 22:6					
1.1/1.05	0.94 (0.11)	11.7 (0.3)	12.8 (1.3)	5.5 (0.3)	4.82 (0.40)
1.5/1.3	0.99 (0.12)	15.1 (0.5)	19.4 (2.5)	6.4 (0.2)	6.17 (0.60)
0.96/1.2	0.72 (0.09)	9.0 (1.0)	11.6 (1.6)	7.0 (0.2)*	5.40 (0.50)
0.98/1.32	0.66 (0.08)	9.2 (0.5)*	12.4 (1.5)	6.2 (0.5)	6.00 (0.50)
III: FA_1 = 20:4, FA_2 = 22:6					
1.2/1.13	0.17 (0.05)	10.6 (0.4)	14.4 (2.0)	4.6 (0.25)	4.73 (0.82)
1.5/1.4	0.17 (0.06)	13.2 (0.5)*	20.7 (2.5)	5.7 (0.2)	5.05 (0.90)
0.99/0.91	0.17 (0.05)	8.2 (0.2)	10.0 (1.2)	5.0 (0.1)	4.40 (0.77)
0.89/1.04	0.136 (0.04)	7.7 (0.2)	9.9 (1.3)	5.8 (0.2)	5.30 (0.80)
IV: FA_1 = 20:4, FA_2 = 18:2					
0.25/0.14	0.47 (0.10)	1.26 (0.10)	1.12 (0.15)	0.95 (0.10)	0.82 (0.20)
0.16/0.13	0.32 (0.07)	0.63 (0.06)	0.70 (0.10)	0.63 (0.06)	0.76 (0.18)
1.4/0.80#	0.62 (1.30)	2.63 (0.25)	3.9 (0.7)	1.77 (0.20)	2.20 (0.40)
0.6/0.63#	0.33 (0.07)	1.01 (0.05)	1.2 (0.2)	1.28 (0.10)	1.40 (0.28)

R is defined as the ratio of FA_2 to FA_1 in the shared binding capacity C_1.
R m_1 is in all cases equal to m_{2o} (*see* Methods Eqs. (6) and (7)). m_1 and m_2 are uptakes calculated from Eqs. (6) to (9).
zero degree determinations, all other determinations are carried out at 37°C.
For abbreviations of fatty acids *see* Table 1.
Numbers in parentheses are SE.
Ghost equilibrium uptakes (M_1 and M_2) of two different fatty acids (^{14}C and ^{3}H labeled) were measured by counting weighted aliquots of ghosts. The uncertainty of M values are inferred from different weighted ghost samples. *Differs from theoretical m values $P < 0.02$. No significant difference in all other cases.
ν_1 and ν_2 are molar ratio of FA_1 and FA_2 to BSA respectively.
ms are calculated and Ms are experimental bindings.

Table 4. Uptakes of palmitic acid and docosahexaenoic acid by red cell membranes with no demonstrable competition

ν_2/ν_1	M_2 nmoles g^{-1}	m_2 nmoles g^{-1}	M_1 nmoles g^{-1}	m_1 nmoles g^{-1}
V: FA_1 = 22:6, FA_2 = 16:0				
0.76/1.1	17.6 (1.5)	19.0 (1.6)	9.6 (1.0)	13.0 (1.1)
0.46/1.07	14.5 (0.1)	13.6 (1.4)	10.2 (0.2)	11.3 (1.0)

For abbreviations *see* Table 3.
(R = 0).

modates a variety of hydrocarbon tails. The conformational changes of the BSA molecule [35] will depend on the shape of the hydrocarbon tail. A small contribution of the entropy to the free energy of transfer from water is associated with a large contribution from the enthalpy (entropy/enthalpy compensation). Thus the large temperature-dependent K_d values of all FAs suggest significant changes in the BSA conformation when FA is bound. In contrast, the virtually temperature-independent K_{dm} values of all unsaturated FA (Table 2) imply a higher contribution of entropy compared to enthalpy in transfer from water to the membrane. As mentioned above, this suggests a large molecular weight of the apolar phase and thus that FA binding to elements involves annular lipids with little conformational change on the part of the protein. The integral proteins have one layer of rapidly exchanging annular phospholipids [31] and an FA hydrocarbon tail may then replace a suitable annular phospholipid. This situation has actually been found in the case of acetylcholine-receptor [5] in order to account for the effect of FA on the receptor function. Accordingly, the different binding capacities with overlapping specificities for different hydrocarbon tails of unsaturated FA can be accounted for by four kinds of elements

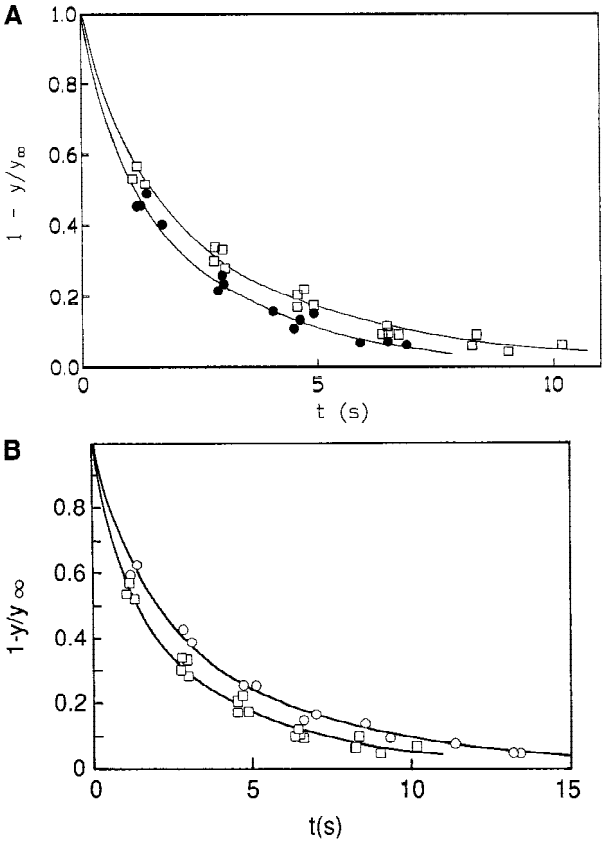

Fig. 5. (*A*) Exchange efflux kinetics of linoleic acid (18:2): □ and of docosahexaenoic acid (22:6): ● from BSA-free ghosts at 0° *C*, $\nu = 0.6$. The extracellular medium contains 0.05% (7.45 μM) BSA. Solid curves show the nonlinear regressions (STATGRAPHIC 5) fitting the data according to Eq. (10) (*see* Materials and Methods). y/y_∞ is the ratio of time-dependent amount of tracer fatty acid in the medium to that at infinite time. (*B*) Exchange efflux kinetics of linoleic acid (18:2) from BSA-free ghosts at 0° *C*, $\nu = 0.6$ at two different BSA concentrations of the medium. □: 0.05% BSA (7.45 μM) and ○: 0.01% BSA (1.5 μM).

Table 5. Transport parameters of fatty acids (FA) from ghosts to bovine serum albumin (BSA) in the medium

FA	*B/E*	k_3 sec^{-1}	k_1 sec^{-1}
16:0	4–5	≈ 0.11	0.0015–0.0026
18:1	0.45 (0.02)	0.067 (0.01)	0.0063 (0.003)
18:2	0.33 (0.03)	0.185 (0.02)	0.14 (0.05)
20:4	0.30 (0.03)	0.39 (0.03)	0.21 (0.02)
22:6	0.45 (0.15)	0.47 (0.024)	0.47 (0.07)

For *B/E*, k_3 and k_1 *see* Appendix.
Exchange efflux kinetics from ghosts at 0°C is analyzed according to the model I Fig 1.

that differ with regard to their annular lipids. However, only some phospholipid head group selection is demonstrated by electron spin resonance for the somewhat disordered annular lipids with restricted motion [31]. Consequently, it is more attractive to assign the origin of the FA-specific affinities and capacities to fatty acyl chains of the membrane phospholipids which fit as annular lipids to the different FA-protein complexes. The hypothesis may be tested in reconstituted systems with an isolated protein and/or by manipulation of the phospholipid composition of a membrane. Thus the membranes may have only one kind of unsaturated FA binding element in a quantity indicated by the binding capacity for 18:1. In contrast, 16:0 binds to a different kind of element resembling albumin in its temperature-dependent binding.

Table 6. Estimated slow rate coefficient (δ^* sec^{-1}) of linoleic acid and docosahexaenoic acid efflux from ghosts filled with bovine serum albumin in concentrations [BSA_i] compared with the expected values δ^* (theor.)(sec^{-1}) calculated according to Eq. (12) in Materials and Methods at different molar ratios of acid to albumin (ν)

[BSA_i] (μM)	ν	$\delta^* = \delta/(1 + \delta/\gamma)sec^{-1}$	δ^* (theor.)sec^{-1}
I: Linoleic acid			
75	1.0	1.00 (0.10)	0.70 (0.10)*
75	0.2	0.58 (0.02)	0.56 (0.06)
30	1.0	2.10 (0.10)	1.70 (0.18)
30	0.2	1.40 (0.05)	1.37 (0.15)
7.5	1.0	6.10 (0.20)	6.20 (0.60)
7.5	1.0	6.90 (0.40)	6.20 (0.60)
II: Docosahexaenoic acid			
30	0.93	9.00 (0.50)	7.60 (1.50)
30	0.18	6.90 (0.10)	6.20 (1.15)
30	0.20	7.00 (0.10)	6.20 (1.15)
7.5	0.95	22.00 (1.50)	26.50 (4.20)
7.5	0.15	15.00 (1.00)	21.00 (3.50)
III: Docosahexaenoic acid + Palmitic acid			
7.5	1 + 1	34.40 (1.00)	40.00 (6.00)
30	1 + 1	13.40 (0.50)	12.80 (2.20)

* Differs from the measured value $P < 0.02$. No significant differences in all other cases.

Tracer Exchange Efflux

The tracer exchange efflux from BSA-free and BSA-filled ghosts is mediated by the membrane binding elements of the pools *B* and *E* situated on the inner and outer surface (*see* models (Fig. 1)). The models imply homogeneous compartments, which is true for the experimental system of a very large number of identical cells, each with a large number of transport elements. In the models, the pools *B* and *E* are in series and the ratio *B/E* is independent on *v* i.e., independent of the occupancies of the binding capacities. Futhermore, plots made for the estimation of *C* and K_{dm}/K_d according to Eq. (3) are all linear, which suggest that only one K_{dm} corresponds to the total capacity (*B* + *E*). The validity of the kinetic model I is demonstrated by the efflux data from studies

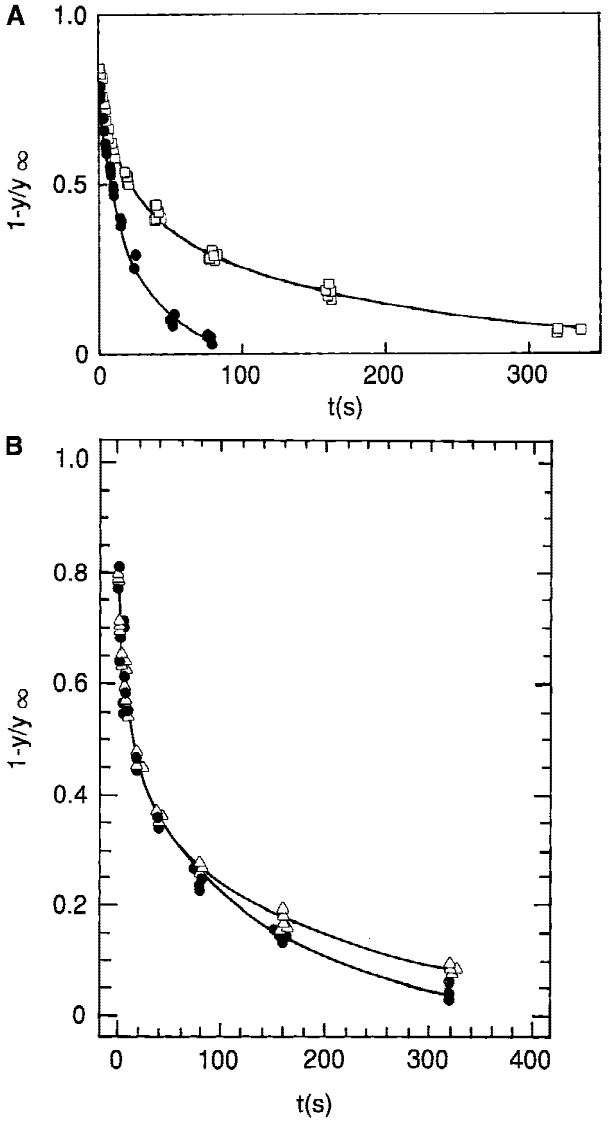

Fig. 6. (*A*) Exchange efflux kinetics of linoleic acid (18:2): □ and of docosahexaenoic acid (22:6): ● from 0.05% BSA-filled ghosts at 0° C, $v = 1$ into the extracellular medium of 0.05% BSA. (*B*) Exchange efflux kinetics of linoleic acid (18:2) from 0.05% BSA-filled ghosts at 0° *C*, $v = 1$, in the presence of arachidonic acid (20:4) at $v = 1$, △ and in the presence of palmitic acid (16:0) at $v = 1$, ● into a medium of 0.05% BSA. Solid curves show the nonlinear regressions (STATGRAPHIC 5) fitting the data according to Eq. (11) (*see* Materials and Methods). y/y_∞ is the ratio of time-dependent amount of tracer fatty acid in the medium to that at infinite time.

of BSA-filled ghosts according to model II, for which the efflux rates can be predicted by the parameters when they are calculated from the results of experiments with BSA-free ghosts (Table 6). The binding capacities are essentially temperature independent (Table 2) and competitive binding measured mainly at 37°C (Tables 3 and 4) is also present at zero degree.

Consequently the *B/E* ratio, which has been calculated from efflux experiments at zero degree, will also be valid at 37°C. As mentioned above, the model predicts the significance of competitive bindings for efflux kinetics shown in Fig. 6*A* and *B*.

The Molecular Mechanism of Translocation of Unsaturated FA

The kinetic model (Fig. 1) corresponds to carrier transport but the question is whether the fact that the models account for the efflux from ghosts both with and without albumin provides unequivocal evidence for a carrier transport. The essential feature of translocation by a carrier is that the ligand remains bound to a protein that changes between two alternative conformations. However, translocation by channeling of the ligand in question with no conformational change of the protein may presumably result equally well in tracer exchange efflux in accordance with the kinetic models. From the opposite binding sites of a protein corresponding to *B* and *E*, FA is released in the neutral form and channeled between *B* and *E* sites through a relatively perturbed annular region [7]. The *E* side of the protein has about twice as many possibilities to bind FA anions electrostatically as the *B* side. Equal fluxes with the same channel translocation rate of neutral FA then means that the frequency of neutral FA release from an *E* site is about half of that from a *B* site. Neutral FA translocated by channeling may exchange with neutral FA in the neutral FA-protein complex. Thus the equilibrium correspondence of inside/outside bound FA described by the models (Fig. 1) may be fulfilled by channeling neutral FA. This suggests that in ghosts only the lipids of the annular region of an integral protein promote the translocation of neutral FA eventually formed at the two sides of the protein. In other cell membranes, an external positively charged protein situated in a suitable bilayer may still promote a saturable unidirectional permeation of the membrane of water-phase FA, although the water/lipid partition of released neutral FA is small. The carrier model is unlikely not only because of the information on the membrane bindings of various unsaturated FAs but also because the rate constant k_3 varies in proportion to the number of double bonds, a finding which supports the channel model. Based on studies of the viscosities of long-chain saturated hydrocarbons [22, 23] it has been suggested that the translatory movements of these molecules are slow due to movements of different chain sections. If the number of double bonds in the hydrocarbon chain is increased, the molecule will consist of fewer independent sections. This must facilitate any translatory movement of the chain. This effect of double bonds is probably more relevant than melting points for the well-known significance of unsaturated FAs for the fluidity of bilayers. As pointed out below there are important studies on

transbilayer movements of monomer neutral FA at 37° [29] reaching equilibrium within 1–2 sec whereas k_3 of 18:1 at 0°C is 0.067 sec^{-1} (Table 5) and about 0.2 sec^{-1} at 20°C with an activation energy of some 50 kJ mol^{-1} (*unpublished*).

Carrier transport has in general been favored to account for saturation kinetics of FA uptake by metabolizing cells. Thus it has been demonstrated that transport of all FAs but no other membrane transports can be partially inhibited by antibodies to a presumptive carrier [21,36]. Another protein, affinity labeled *in situ* [26,27] is likely to play a part in FA translocation. The transport is inhibitied by about 60% by the blocking agent and this observation is compatible with the idea that several proteins may participate in FA transport even if only some segments of them are required for the transport. Incomplete block of FA transport by a presumptive complete block of a carrier is usually explained by a parallel bilayer permeability of FA. No such bilayer permeability is present in the ghost membrane, which has a typical bilayer [2], because our binding data are in agreement with efflux data in accordance with the described models.

Liposome Binding and permeation of FA. A critical review.

Based upon studies of the interactions between FAs and various kinds of liposomes it has been suggested, that the capacity for rapid transport of FA through biological membranes can be assigned to the lipid bilayer itself. Different liposome preparations differ apparently with regard to their binding and transfer of FAs. In one important study [32] a saturable uptake of FA monomers (anions at neutral pH) is ensured by offering FA bound to albumin in molar ratio $v \leqslant 3$. The uptake was measured in small unilamellar vesicles with 10% lactocerebroside/egg lecithin after precipitation with lectin ([32] Fig. 2). If the data are considered as a plot of FA/lipid molar ratios *vs.* water-phase monomer concentrations [FA] according to $[FA]/K_d = v/(3-v)$, They suggest a maximal uptake of about 1 mol% with a K_{dm} very similar to that of ghost membrane binding of 16:0 calculated from the K_d value given in Table 2. This uptake of the FA anion into liposomes lacking protein suggests that egg lecithin may be arranged as positively charged microdomains, as demonstrated by Jones [28].

Selective uptake and rapid transfer of the neutral monomers has, however, been demonstrated by Kamp and Hamilton [29]. These authors show that the pH of the intraliposomal aqueous phase decreases rapidly to a new lower equilibrium value when FA is added to the BSA-containing medium. Knowing the FA anion concentration determined by v and the concentration of neutral FA calculated from the pK_a, it is possible to calculate that the inner to outer equilibrium pH may reflect membrane partitions of neutral FA dissociated in equivalent numbers of protons and anions within the liposomes until neutral FA is the same as outside. Such a flux of neutral FA from FA in water at neutral pH and $v = 1.5$ through the ghosts bilayer is in theory negligible, because the saturable uptake of FA anions is several orders of magnitude greater than the uptake of neutral FA by phase partition. This is not compensated by a difference in rates of membrane permeation as this rate of neutral FA is not greatly different from that of FA translocation through the ghost membrane as mentioned above. A higher resolution time of the rate of liposome water acidification is obtained by stop flow studies [30]. However, in these studies ethanolic solutions of FAs was added to the aqueous phase and the FA concentration was in the μM range where dimers and higher aggregates are formed [34,38]. These are much more hydrophobic than monomer FA and the observed fast rate of acidification is consequently of doubtful significance. A comparable equilibrium study of FA uptake by liposomes [3] demonstrated the expected phase partition with nearly quantitative uptake by the liposomes. Such high concentration of FA changes the liposomal structure completely.

Conclusions

A Hypothesis on Permeation of Unsaturated FAs

(i) The maximum number per cell of binding elements for unsaturated FAs (that determined for 18:1) is approximately equal to the number of glucose carriers. Different fractions of the elements bind the different unsaturated FAs.

(ii) The transfers of unsaturated FA from water to membrane elements are largely determined by the entropic contribution to the free energy change. The competitive bindings can be explained as competitive binding of the FA anions to protonated amino acid residues of a protein, whereas the hydrocarbon chains are immersed into the annular lipids. The fraction of the protein that binds any unsaturated FA may be limited by the fraction of membrane lipids which are capable of fitting to the FA-protein complex as annular lipids.

(iii) The asymmetric inside/outside distribution is similar for all four unsaturated FAs and the kinetics of their transfer is compatible with carrier kinetics. In this case the equilibrium FA distribution reflects the two rates by which the bound ligand is translocated from one side to the other. However, carrier kinetics is incompatible with two observations on unsaturated FAs: (*a*) the rates of translocation are proportional to the number of

FA double bonds and (*b*) the binding capacities for various FAs are different and partially shared. We suggest that the elements binding water-phase FA consist of an integral protein with annular lipids that are different for different unsaturated FAs. The protein has about twice as many binding sites at the outside than at the inside. Bound anionic FA is reversibly protonated and neutral FAs are channeled through annular lipids between the inside and outside binding sites. Thus transport of unsaturated FAs may combine the ''saturable transport kinetics'' and the lipid bilayer permeation of neutral FA, which we have referred to in the discussion.

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Appendix

List of Symbols

Symbol	Definition	Unit
K_d	equilibrium dissociation constant for BSA-FA complex	nM
N	number of equivalent BSA binding sites	
ν	molar ratio of BSA-bound FA to BSA	
K_{dm}	equilibrium constant for FA dissociation from ghost membranes	nM
C	binding capacity of membranes of 1-g ghosts	nmoles
B/E	ratio of FA bound to the inner surface to FA bound to the outer surface of ghost membranes	
M	experimental ghost membrane bound FA	nmoles g^{-1}
m	theoretical ghost membrane bound FA	nmoles g^{-1}
R	ratio of FA_2 to FA_1 in the binding capacity C_1 (for FA_1)	
Q	ratio of FA in transport pool B to FA bound to BSA within ghosts	
δ	the minor rate coefficient of FA exchange efflux according to Eq. (11)	sec^{-1}
γ	the major rate coefficient of FA exchange efflux according to Eq. (11)	sec^{-1}
δ^*	an experimental modified rate coefficient equal to $\delta/(1+\delta/\gamma)$	sec^{-1}
δ^* *(theor.)*	a theoretical rate coefficient defined by Eq. (12)	sec^{-1}
C_a	total FA concentration in solution after equilibration of ghosts with solution containing radioactive FA	dpm ml^{-1}
C_w	water-phase concentration of FA	dpm ml^{-1}
S_a	FA-specific activity	dpm $nmoles^{-1}$
k_1	dissociation rate constant of FA-BSA complex	sec^{-1}
k_1^*	integrated effective mean dissociation constant of FA-BSA complex within ghosts	sec^{-1}
k_5	rate constant of unidirectional flow from E to the adjacent water-phase (glycocalyx space, See Fig. 1)	
k_5^*	rate constant of unidirectional flow from E to BSA in the medium through the water-phase	sec^{-1}
k_3	rate constant of unidirectional flow through the membrane from B to E	sec^{-1}
k_3^*	rate constant of unidirectional flow from B to BSA in the medium through the E compartment	sec^{-1}
S	surface area of 1-g ghosts (containing $9 \cdot 10^9$ ghosts)	μm^2
D	diffusion coefficient of FA	$\mu m^2 \cdot sec^{-1}$
R_D	diffusion resistance of the unstirred volume surrounding 1-g ghosts	$sec \cdot ml^{-1}$