Inactive fatty acids are unable to flip-flop across the lipid bilayer

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Abstract Free fatty acids (FA) were found which did not acidify liposome interior. This is interpreted as their inability to rapidly flip-flop across the lipid bilayer. However, they were able to partition in lipids as detected directly using HPLC or from the shift of their equilibrium binding to acrylodated intestinal binding protein (ADIFAB) in the presence of vesicles. Various bipolar FA, such as 12-hydroxylauric acid, dicarboxylic acids, or FA with benzene ring at the tail were found to be inactive in this way. A phenomenon of shielding, where an additional alkyl chain or non-polar group can restore the flip-flop activity, is described.

Key words: Fatty acid flip-flop; Fatty acid structure; H^+ transport

1. Introduction

H^+ permeability of pure lipid bilayers (the net H^+ permeability) or of biological membranes (proton leak), was extensively studied namely in bioenergetics [1–4], because of its inherent importance in the chemiosmotic theory. One reason for the excessive H^+ permeability of biomembranes when compared to artificial lipid bilayers has been found in the frequent presence of unesterified fatty acids (FA) in membranes [5]. Indeed, long-chain FA are capable of rapid nonionic flip-flop across biological membranes [6,7]. Nevertheless, a FA-induced uncoupling in mitochondria and other coupling membranes is not fully explained by this phenomenon either [8]. Skulachev [9] hypothesized that membrane proteins may conduct FA anions [10] while protonated FA flip back across the lipid bilayer, thus carrying H^+. The FA cycling then mimicks the classic uncoupler cycling [10].

A question whether both the protonated and the negatively charged FA are equally able to flip-flop across the lipid bilayer has been solved. Kamp and Hamilton [6,7] have shown that only the protonated FA can flip-flop very rapidly, while flip-flop or diffusion of the negatively charged FA was several orders of magnitude slower. Rates of flip-flop were inferred from the internal acidification of liposomes upon addition of FA. Interestingly, the FA with bulky groups such as 5-DOXYL-stearic acid, 1-pyrenenonanoic acid exhibited the same fast flip-flop with $t_{1/2} < 1$ s [7]. Only flip-flop of 12-(9-anthroyloxyl)stearic acid proceeded with $t_{1/2}$ of 200 s [7].

This study deals with the structural features of FA that affect the FA's ability to flip-flop. We found several FA derivatives which were unable to flip-flop, at least in the time course of our experiments. Their flip-flop rates, if they exist at all, must be corresponding to $t_{1/2} > 30$ min. We named these FA 'inactive' and recommend them as a tool for studying the FA-induced uncoupling.

2. Material and methods

The fluorescent probes SPQ and ADIFAB were purchased from Molecular Probes (Eugene, OR). Various derivatives of fatty acids were purchased from Sigma and Fluka (Germany) or Lancaster (UK). Egg yolk L-α-phosphatidylcholine, L-α-phosphatidic acid, cardiolipin and other materials for liposome preparation were from the sources as described elsewhere [10].

2.1. Measurement of the net H^+ influx into liposomes

We used the 'SPQ quenching' method of Orosz and Garlid [11] to monitor the internal acidification of vesicles resulting from the flip of added FA. SPQ is quenched by TES^- anion, but not by TES zwitterion, which allows for monitoring of H^+ fluxes after rigorous calibration by KOH in the presence of nigericin [11]. FA were added as μl aliquots of stock solutions in ethanol. Liposomes (25 μl per assay) prepared by the detergent (Octyl-POE) removal method with Bio-Beads SM2 contained 84.4 mM TEA-SO_4, 28.85 mM TES (TEA-salt), pH 7.2, and 0.6 mM TEA-EGTA. External medium contained 84.4 mM K_2SO_4, 50 mM TES (TEA-salt), pH 7.2, and 1.5 mM TEA-EGTA. Valinomycin (0.1 μM) was added at 25-30 s in order to test for the H^+ leak.

2.2. Measurement of FA partitioning between water and liposomes

FA (8 μmol) was added to liposomes (40 mg lipids) and the aqueous portion of FA was roughly estimated as the difference between the original (total) amount of FA and the amount retained in the vesicles after gel filtration on Sephadex G25-300, using spin-columns. The amount of FA was analyzed after sample derivatization by phenylacylbromide [12] on an HPLC system (Waters, USA) with an automated gradient controller, Model 510 pumps, and W490E absorbance detector (242 nm) using a 10 μM Nucleosil 250×4 mm column (Macherey-Nagel, Düren, Germany) at 46°C. Elution was done by a linear gradient of H_2O (A)/acetonitrile (B) from 70% B to 100% B at 30 min with a flow rate of 2 ml/min.

2.3. Measurement of FA partitioning between water and liposomes: ADIFAB method

To estimate free FA we used a decrease in the slope of a plot of the ADIFAB fluorescence ratio, R, at 505–432 nm versus total amount of FA, $[FA]_{total}$ [13]. Excitation was set at 477 nm. Experiments were performed on a SLM 8000C fluorometer. The assay medium contained 150 mM NaCl, 1 mM TEA-EGTA, 10 mM Tris-HCl, pH 8, with 0.2 μM ADIFAB [14,15]. The amount of free FA was calculated from the experimentally obtained ratios as described [14,15]:

$$[FA]_{free} = K_d \cdot Q \cdot (R - R_0)/(R_{max} - R) \quad (1)$$

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Abbreviations: ADIFAB, acrylodated intestinal fatty acid binding protein; DOXYL, 4,4-dimethyl-3-oxazolinyloxyl-; FA, free fatty acids (non-esterified); Octyl-POE, octylpentaoxy-ethylene; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; TEA, tetraethylammonium-; TES, *N*-tris [hydroxymethyl]methyl-2-aminoethane-sulfonic acid; TNP, trinitrophenyl-

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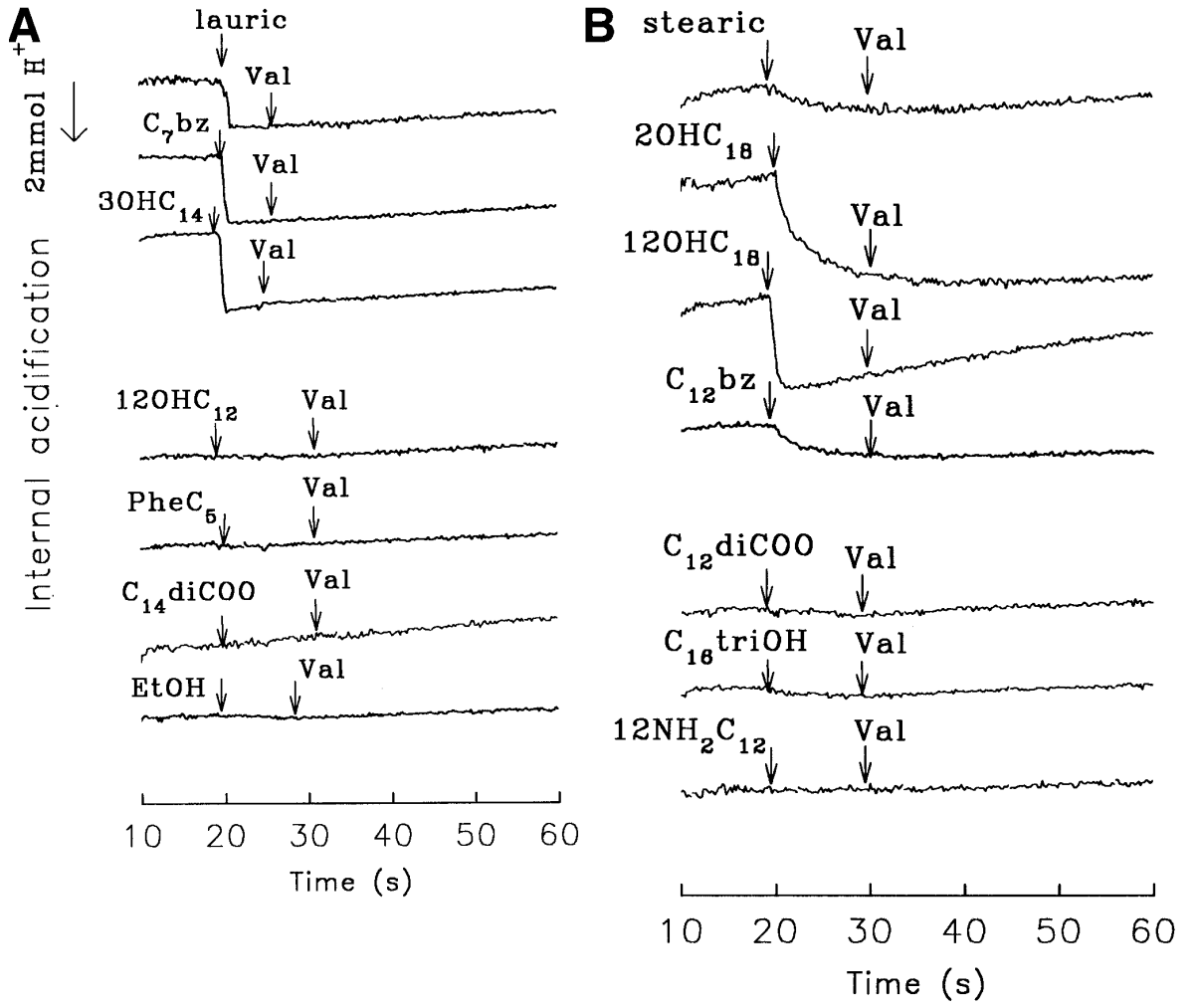

Fig. 1. Liposome interior acidification is caused by the 'active' fatty acids but not by the 'inactive' fatty acids. *Active FA*: The upper-half contains traces obtained in the presence of 50 μM lauric, heptylbenzoic (C_7bz), 3-hydroxymyristic ($3OHC_{14}$), stearic, 2-hydroxystearic ($2OHC_{18}$), 12-hydroxystearic $12OHC_{18}$) and dodecyloxybenzoic (C_{12}bz) acids. The lower-half contains traces obtained in the presence of the *inactive FA*: 12-hydroxylauric($12OHC_{12}$), phenylvaleric ($PheC_5$), tetradecanedioic (C_{14}diCOO), dodecanedioic (C_{12}diCOO), 9,10,16-trihydroxypalmitic (i.e. aleuritic, C_{16}triOH) and 12-aminolauric ($12NH_2C_{12}$) acids. The bottom trace of the left panel was obtained in the presence of ethanol (EtOH) only. The amount of ethanol was the same as the amount added with FA. 0.1 M valinomycin (Val) was added at 25 or 30 s to test for the H^+ leak.

where K_d is an equilibrium constant for binding of the given FA to ADIFAB; Q is the ratio of fluorescence at 432 nm at zero and saturating FA concentrations; R_0 and R_{max} are the corresponding R ratios at zero and saturating FA concentrations, respectively. We had to calculate our own parameters Q (14.5) and R_{max} (5) while using Marquardt algorithm to fit the Eq. 4 in [15] onto our data obtained with oleic acid. This procedure yielded K_d values to those published for oleic acid [15] binding to ADIFAB (0.39 μM, 0.22 μM, 2 experiments). Eq. 1 has been used also to calculate $[FA]^L_{free}$ at the equilibrium shifted due to the addition of liposomes. The respective K_ds calculated from the data of the experiments without liposomes were used. We then evaluated the partition coefficient K_p from the data around K_d according Anel et al. [14] as follows:

$$K_p = (V_a/V_m) \cdot (1/[FA]^L_{free}) \cdot ([FA]_{total} - [FA]^L_{free}) \quad (2)$$

where V_a/V_m is the ratio of volumes of the aqueous and membrane phases. V_m/V_a of 0.001 per 1 mM phospholipids was used [14].

3. Results and discussion

In the top half of Fig. 1 is illustrated the internal acidification in liposomes exposed to a K^+ gradient upon addition of different FA at 50 μM concentrations. An electrochemical K^+ gradient of approximately 180 mV across the liposomal membrane was present in all experiments. Net H^+ flux in vesicles was monitored via quenching of SPQ by TES^- anion [11]. According to Kamp and Hamilton [6,7], the acidification of vesicle interior can be interpreted as flip of the protonated FA from the outer to the inner lipid leaflet of the membrane. The scheme in Fig. 2 shows the possible intermediate non-equilibrium states involved and explains why an internal acidification can be an indication of FA flip into the inner lipid leaflet. The addition of 0.1 μM valinomycin in the presence of FA caused negligible alkalization of vesicle interior. The FA-induced H^+ leak is therefore very low [10] and equal to the H^+ leak existing in the absence of FA (cf., Fig. 1, bottom trace with only ethanol added), in spite of the established K-diffusion potential of over 180 mV. The leak magnitudes were between 0.007 and 0.03 nmol H^+ s^{-1} with the inactive FA and 0.016–0.04 nmol H^+ s^{-1} with the active FA. For comparison, the H^+ leak in the presence of ethanol was 0.013–0.025 nmol H^+ s^{-1}. Extrapolating to zero mV [16], permeability coefficients

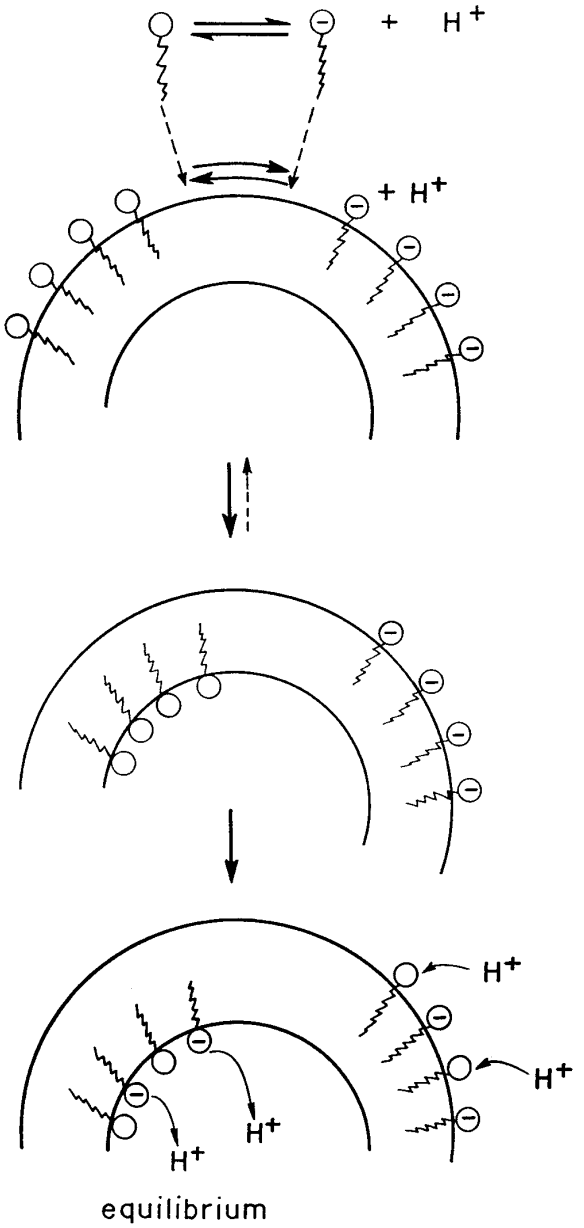

Fig. 2. The equilibrium reached upon external addition of fatty acid to liposomes. One may simply realize, why interior acidification contradicts to the flip-flop of both anionic and protonated FA molecules. In this case, half of each species which are originally distributed according acid-base equilibrium would flip into the inner lipid leaflet and the inner acid-base equilibrium would be set automatically if external pH was identical to the internal pH. Hence, there is no need for the interior acidification. Different situation occurs, however, when only neutral FA can flip-flop. In order to establish equilibrium between the two leaflets of a bilayer, the majority of nonionic FA which partitioned into the outer leaflet must flip into the inner leaflet. Consequently, a new acid-base equilibrium must be established in the vesicle interior, which leads to the internal H^+ release.

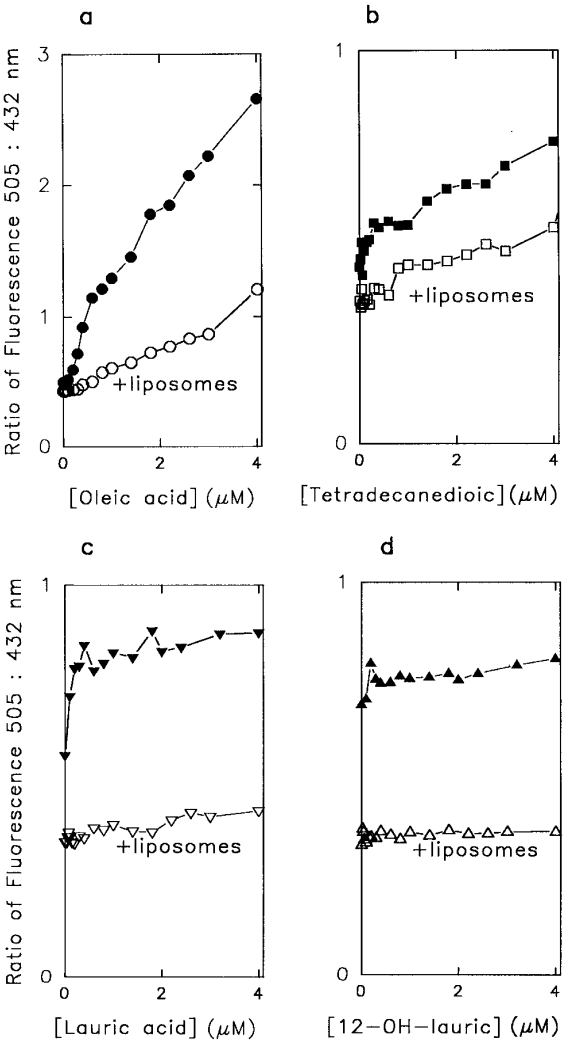

Fig. 3. Fatty acid binding to ADIFAB in the presence and absence of liposome. Binding of FA to ADIFAB was measured in the absence (filled symbols) and presence of liposomes (20 μM lipid, open symbols) for (a) oleic acid; (b) tetradecanedioic acid; (c) lauric acid; (d) 12-hydroxylauric acid. The characteric saturation binding reflected by a plot of fluorescence ratio, R, at 505–432 nm vs. total amount of the added FA, $[FA]_{total}$, is shifted upon the addition of liposomes, thus indicating FA partitioning into the lipid bilayer. Measurements were done with the sodium salts in FA in a medium containing 150 mM NaCl, 1 mM TEA-EGTA, 10 mM Tris-HCl, pH 8, with 0.2 μM ADIFAB [14,15]. The derived K_d values are listed in the Table 2, as well as the calculated partition coefficients K_p.

derived from these data were 1.10^{-4} to 4.10^{-4} cm·s^{-1}. H^+ leak was significantly higher in the presence of 12-hydroxystearic (Fig. 1) and hexadecanedioic acids (not shown).

All physiologically abundant FA and their derivatives demonstrated the ability to acidify vesicle interior with a $t_{1/2}$ well below 1 s, as reported [7]. Nevertheless, we found several FA derivatives (Fig. 1 and Table 1), which did not acidify vesicle interior even if present in concentrations of 200 μM. No significant changes in pH_{in} were observed for up to 5 min. We conclude that these FA were unable to flip-flop with a significant rate and we call them 'inactive FA' to distinct them from the 'active FA' which do posses the ability of fast flip-flop.

Typically, the inactive were FA with a polar group in the 'tail' terminal (ω) position. Besides the 12-hydroxylauric acid, the 12-aminolauric[2] and dodecanedioic acids which were inactive in the laurate group, the tetradecanedioic acid was inactive in the myristate group. One would assume presence of

[2] NH_2 group has low polarity, but might form NH_3^+.

Table 1
Flip-flop of various fatty acids across the lipid bilayer as indicated by internal acidification in liposomes

Compound	Extent of flip-flop acidification[a] (total nmol H^+ released per 100 nmol FA added)	$t_{1/2}$ half-time of flip-flop (s)	
Active fatty acids			
Lauric ($n=7$)	2.54	0.387	
2-Hydroxylauric	1.76	0.5	
12-Bromo-lauric	2.57	1.0	
12-TNT-lauric	2.20[c]	4.0[c]	
Myristic	4.20	0.4	
2-Hydroxymyristic	5.67	0.1	
3-Hydroxymyristic ($n=3$)	4.57	0.28	
Palmitic	3.34	0.45	
2-Hydroxypalmitic	8.20	0.55	
Stearic	0.91	2.5	
2-Hydroxystearic	4.64	1.6	
12-Hydroxystearic	4.28	0.5	
Heptylbenzoic ($n=4$)	3.92	0.3	
Dodecyloxybenzoic	1.12	2.4	
16-Hydroxypalmitic	1.68	0.5	
Hexadecanedioic[b] ($n=4$)	0.44	1.7	
Inactive fatty acids		with 50 μM [d]	with 200 μM [d]
12-Hydroxylauric ($n=4$)	0.007	> 60 min	> 60 min
12-Aminolauric ($n=3$)	0.04	> 60 min	> 30 min
Dodecanedioic	0.03	> 2 min	> 2 min
Tetradecanedioic	0.015	>200 min	
Phenylhexanoic	0.07	>200 min	>200 min
Phenylvaleric ($n=3$)	0.005	>200 min	>200 min
9,10,16-Trihydroxypalmitic	0.03		> 4 min
Biphenyl-2-carboxylic	0.08	> 30 min	>200 min
3,3-Diphenylpropionic	0.09	>200 min	> 60 min
β-Naphtoic	0.003	> 30 min	> 60 min

Experiments were performed in duplicate if not indicated otherwise (n, number of measurements listed in parentheses) with 50 μM FA added or with 50 and 200 μM in the case of the inactive FA.
[a]With 100 nmol FA added, this is numerically equal to % of FA molecules partitioning in lipid bilayer. In case of the inactive FA the extent is not related to partitioning.
[b]Increased H^+ leak.
[c]Measured with 10 μM, separately calibrated and accounted for shift due to absorbance.
[d]Calculated with regards to extent of H^+ release for lauric acid.

half protonated molecules at pH 7.2 in the case of dicarboxylic acids that could mimic the monovalent laurate (myristate) anions. The negligible acidification suggested the second protonated carboxyl group represents entity so polar it prevents the flip-flop. The less polar bromo group was tolerated since 12-bromododecanoic acid flips rapidly (Table 1).

All 2-hydroxy-FA tested were active. With exception of 2-hydroxylauric acid, all 2-hydroxy-FA exhibited faster rates and higher extent of H^+ release than their basic analogs (Table 1). The 2-hydroxymyristic acid was the fastest studied with 3-hydroxymyristic acid only slightly slower. Stearic acid exhibited a low extent of H^+ release with a $t_{1/2}$ of 2.5 s. Interestingly, 12-hydroxystearic acid was found also to be active and fastest among stearate series (Fig. 1, Table 1). One can explain it by a shielding effect of the tail hexyl chain on the hydroxy group. Such shielding eliminates the original bipolar character of the hydroxylated C12-aliphatic chain. Also bipolar 16-hydroxypalmitic acid exhibited a fast acidification of moderate extent (Table 1), thus behaving distinctly from the shorter 'tail'-substituted FA. Hexadecanedioic acid induced a

Table 2
Partitioning of the selected fatty acids into the lipid bilayer as evaluated using ADIFAB

Fatty acid	Binding to ADIFAB K_d (μM)	Partition coefficient K_p
Oleic acid	0.39	
	0.22	51 000
Myristic acid	1.44	81 000
Lauric acid	1.2	
	4.5	220 000
Tetradecanedioic acid	2.33	66 000
12-Hydroxylauric acid	9	50 000
3-Hydroxymyristic acid	20	11 000
2-Hydroxylauric acid	infinite[a]	

Binding of FA to ADIFAB was measured in the absence and presence of liposomes (18 μM) formed from L-α-phosphatidylcholine, cardiolipin and 1% L-α-phosphatidic acid and the corresponding K_d values and partition coefficients were calculated as described in Section 2.
[a]2-Hydroxy-lauric acid did not bind to ADIFAB and addition of liposomes did not change the ADIFAB fluorescence at any 2-hydroxy-lauric acid concentration. This provides evidence that liposomes per se are not affecting ADIFAB.

small drop in the pH_{in} (Table 1), but it did induce a high H^+ leak so we cannot exclude the lytic character of the effect. We further ascribed 9,10,16-trihydroxypalmitic acid (aleuritic acid) as inactive (Fig. 1 and Table 1), because it caused a negligible acidification, with an estimated $t_{1/2}$ over 4 min.

The derivatives bearing benzene ring, i.e. phenylvaleric and phenylhexanoic acids and derivatives with two benzene rings such as biphenylcarboxylic acid, 3,3-diphenylpropionic acid and β-napthtoic acid are inactive (Fig. 1 and Table 1). The phenyl group with lesser polarity than the hydroxy-group probably prevents the transmembrane flip-flop due to bulkiness and planar structure of the benzene ring. It is a benzene ring at the tail which is not tolerated, since dodecyloxy-benzoic and heptylbenzoic acids are active. The latter is very potent and belongs to the fastest FA studied (Fig. 1 and Table 1). FA bearing bulky but overall less polar groups such as 12-TNP-lauric, 12-(4-azido-2-nitrophenyl) aminododecanoic [17], 12-DOXYL- and 5-DOXYL-stearic acids [7,18] were found to flip-flop with a fast rate. The first two are another examples of a shielding effect. The NO_2 group substitutions on the aromatic ring convert it to an entity tolerated by flip-flop.

Inability of FA to exert a fast flip-flop is a very important property. The trivial explanation for lack of flip-flop is the lack of partitioning of the inactive FA into the lipid bilayer. We have, however, observed otherwise. Using HPLC detection we showed that 15.5% of 12-hydroxylauric acid is retained in the liposomes after their passage through the Sephadex G25-300 column, as compared to 27.8% of lauric acid. Also Fig. 3 demonstrates the partitioning of 12-hydroxylauric and tetradecanedioic acids into liposomes (Fig. 3d,b) as compared to their active counterparts, oleic and lauric acid (Fig. 3a,c). In all cases, including the 12-hydroxylauric and tetradecanedioic acids, it is shown that the equilibrium between ADIFAB and aqueous phase is strongly disturbed by the presence of liposomes (20 μM lipid concentration). Partitioning into lipids is also implied from calculations of the respective partition coefficients K_p (Table 2). The evaluated K_d values for FA binding to ADIFAB (Table 2) were not directly related to the FA ability to flip-flop. For example, the binding of α- or β-hydroxy-FA to ADIFAB was very poor.

At present we are unaware of physicochemical mechanism which prevents some FA derivatives from a rapid flip-flop across the lipid bilayer. Considering that even pyrenenonanoic acid exhibited flip-flop with $t_{1/2} < 1$ s [7], inability of some bipolar FA to flip-flop in the time scale of minutes is surprising. The only FA previously reported to exhibit a slow flip-flop was 12-(9-anthroyloxy)-stearic acid ($t_{1/2}$ of 200 s). A rather trivial explanation for the inability of flip-flop would be that partitioning of the inactive FA into the lipid phase is very low. We excluded this possibility by showing that the inactive FA *do* partition into the lipid bilayer, as detected from their interactions with ADIFAB. Therefore, we suggest that rather a specific conformation of the inactive FA in the membrane is the cause for lack of rapid flip-flop. Perhaps a U-shape conformation in the case of bipolar FA or a 'dumbbell' shape of the 'tail'-phenyl-substituted FA. The properties of the inactive FA with regard to the lipid bilayer deserve further studies by physicochemical methods. We also strongly recommend to use the inactive FA as controls for studying the FA-induced phenomena such as uncoupling, activation of proteins, etc.

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