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Partition of Fatty Acids and Fluorescent Fatty Acids into Membranes†

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ABSTRACT: We have measured the membrane/water partition of free fatty acids, of a fluorescent analogue, and of chlorpromazine into plasma membranes from lymphoma, platelets, red cells, and liposomes of egg phosphatidylcholine. Three different methods were used: hygroscopic desorption [Conrad, M. J., & Singer, S. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5202–5206; Conrad, M. J., & Singer, S. J. (1981) *Biochemistry 20*, 808–818], centrifugation, and fluorescence titration. The partition coefficients for chlorpromazine and all fatty acid/membrane combinations were in the range 10^2–10^5. No significant partition difference was observed between liposomes and plasma membranes. These results suggest, therefore, that fatty acids and chlorpromazine can readily partition into artificial and cell plasma membranes.

Much of our understanding of membrane structure and function has been inferred from the interaction of amphipathic molecules with the lipid bilayer of the membrane (Marsh, 1981; Yguerabide & Foster, 1981). In most of these studies, it is assumed that the hydrophobic portion of the molecule or probe monitors the internal structure of the membrane. It follows that the hydrophobic portion of the amphipath partitions into the bilayer so that it is shielded from the aqueous environment. With the exception of artificial liposomes (Lesslauer et al., 1972; Podo & Blaisie, 1977), however, direct proof (for example by X-ray or NMR methods) of the internal location of the probes is lacking.

Recently, Conrad & Singer (1979, 1981) have called into question one of the basic tenets of membrane probe studies. These authors raised the intriguing possibility that although amphipaths can partition into artificial liposomes, they are excluded from the internal regions of biological membranes. Instead, they suggested the generally observed associations of probes with membranes result from the interaction of amphipathic micelles with the surface. It was hypothesized that virtually all indirect observations of membrane/probe associations could be interpreted in terms of these micelles.

Conrad & Singer (1981) argued that centrifugation, filtration, or spectroscopic probe signal enhancement methods are inadequate to determine the membrane/water partition of amphipaths, since in attempting to separate membrane-associated from free amphipath no provision is made to eliminate the formation of membrane-bound micelles. To overcome this problem, they developed a technique called hygroscopic desorption in which the aqueous phase is removed from the membranes and replaced by air. Using this technique, it was found that four amphipaths, chlorpromazine, methyl chlorpromazine, 2,4-dinitrophenol, and 1-decanol, exhibited large partition coefficients into artificial liposomes. The partition into red cell and lymphoma plasma membranes, however, was at least 10^4 times lower and was, in fact, consistent with zero. With these observations in mind, Conrad & Singer (1981) suggested that no amphipath, including fatty

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acid analogues, which are often used as membrane probes, could overcome the (presumed) very high lateral pressures of biological membranes in order to insert into the hydrocarbon region.

In this paper, we have studied the partition of fatty acids and a fluorescent analogue into lymphoma, platelet and red cell membranes, and liposomes of phosphatidylcholine by using three techniques: hygroscopic desorption, centrifugation, and fluorescence titration. We find that fatty acids and the fluorescent analogues do partition into membranes and that results of the various techniques are in substantial agreement.

Materials and Methods

Stearic, linoleic, palmitic, and oleic fatty acids were purchased from Avanti Chemicals, and 12-(9-anthroyloxy)stearic acid (12-AS) was from Molecular Probes. Stock solutions of the fatty acids at concentrations between 5 and 35 mM were prepared in ethanol. ^{14}C-Labeled analogues of the fatty acids, [^{3}H]inulin, [^{14}C]dipalmitoylphosphatidylcholine, and [^{14}C]-chlorpromazine were purchased from New England Nuclear. Egg phosphatidylcholine was purchased from Avanti Chemicals and used without further purification. The buffer used in all experiments was 10 mM tris(hydroxymethyl)aminomethane (Tris), 130 mM NaCl, and 0.2% NaN_3, adjusted to pH 7.4.

Liposomes and Membranes. Two kinds of liposomes were prepared from egg phosphatidylcholine with trace amounts of [^{14}C]dipalmitoylphosphatidylcholine. Multilamellar vesicles (MLVs) were prepared by vortex mixing of an aqueous dispersion of the lipid, as described previously (Klausner et al., 1980), and small highly sonicated unilamellar vesicles were prepared by the method of Huang & Thompson (1974). Lymphoma (CH1) plasma membranes were prepared essentially by the method of Lemmonier et al. (1978). The CH1 cell line was grown in vivo by using either male or female B6AFI/J mice (Jackson Laboratories). After cells from these mice were washed, they were resuspended to a concentration of 10^8 cells/mL and lysed by using nitrogen bomb cavitation. Plasma membrane was purified on a discontinuous 25–37% sucrose gradient and stored at −20 °C. Human red cell ghosts were prepared from fresh blood by the method of Dodge et al. (1963). Ghost vesicles enriched in the anion-transport protein band 3 were prepared essentially by the method of Wolosin et al. (1976). Phospholipid content of the purified plasma membranes was assayed by the method of Bartlett (1959).

Amphipath Addition. Small volumes (<0.5%) of fatty acids concentrated in ethanolic solutions were added to membranes or vesicles while vortex mixing. The mixture was allowed to incubate for about 30 min at room temperature before filtration or centrifugation. Shorter incubations (5–15 min) were employed for the fluorescence titration measurements using 12-AS, since, as indicated by the rate of increase of fluorescence intensity, equilibrium is reached within 1–2 min.

Radioactivity. Radioactivity was determined by scintillation in a Soluene 350-Dimilume 30 (Packard Instruments) cocktail using a Beckman LS-333 counter, and corrections for quenching were made with calibrated standards.

Fluorescence. Fluorescence intensities were measured by using a Perkin-Elmer MPF-2A spectrofluorometer with excitation and emission slits at 4 nm. The excitation and emission wavelengths, respectively (in nanometers), were 383 and 440 for 12-AS, 290 and 340 for Trp, and 352 and 440 for quinine sulfate. Intensities were measured in 10-mm path-length cuvettes and, after corrections for inner filter effects, were taken as the peak height at the emission wavelength. All fluorescence intensities were evaluated relative to a solution of quinine sulfate in 0.1 N H_2SO_4.

Hygroscopic Desorption. Hygroscopic desorption measurements were carried out essentially as described in Conrad & Singer (1981). Three filters were mounted in a Sartorius SM 16-3-16 filtration unit. The top filter, which served to retain membrane, was a polycarbonate film 5 μm thick (Nuclepore Corp. 113602). [Conrad & Singer (1981) also used a 2 μm thick filter from Mobay Chemical Corp.] The middle filter, whose function was to keep the underside of the top filter from being wetted by the bottom filter, was either a grade 30 or a 1-HV type inert glass filter of 100-μm thickness (Schleicher & Schuell). The bottom filter was a pad of absorbent white cellulose of 1.0–1.5-mm thickness (either the Millipore AP 1003700 or the Schleicher & Schuell 470 cellulose filter). Either the grade 30 and AP 1003700 filters or the Schleicher & Schuell 1-HV and 470 filters were used together with the Nuclepore film. Both combinations yielded equivalent results. The filters were treated according to Conrad & Singer (1981) with either of two surfactants, poly(vinylpyrrolidone) (Sigma, PVP-400) or poly(vinyl alcohol) (Polysciences, Inc., no. 15133), and gave equivalent results. The polycarbonate film was conditioned by washing in 10–15% acetic acid for about 30 min. The film was then rinsed with deionized H_2O and incubated overnight in a 1% solution (ethanol/H_2O, 1/6 v/v) of either poly(vinylpyrrolidone) or poly(vinyl alcohol). The film was then rinsed with deionized water, dried, and used immediately.

Samples (1–1.5 mL) were filtered from 15 to 30 min under vacuum. The radioactivity (or 12-AS fluorescence intensity) was determined in the prefiltrate sample (C_T) and in the sample remaining on the filter (C_F). In the case of 12-AS, the fluorophore was extracted from the filter by washing in 3 mL of ethanol. Nonspecific retention of the aqueous phase on the filter was monitored by including $^{3}H_2O$ or [^{3}H]inulin in the sample.

The membrane/water partition coefficient (K_p) was evaluated according to

$$K_p = \frac{C_F V_S}{C_S V_m} \tag{1}$$

in which C_S ($=C_T - C_F$) is the supernatant counting rate, V_m is the volume of the membrane, and V_S is the volume of the supernatant phase (approximate total sample volume). V_m was estimated as 10^{-3} mL/mM phospholipid by assuming that each phospholipid occupies 70 × 25 Å^3 and that the cholesterol and protein contribute an additional 30%.

Centrifugation. Samples were centrifuged in a Beckman SW 50.1 rotor at 100000*g* for 30 min. Radioactivity, phospholipid content, and Trp fluorescence intensities were determined for the precentrifuged sample. The concentration of membrane in the pellet and supernatant was determined from their Trp intensities by using the separately measured relationship between Trp fluorescence and phosphate concentration. Partition coefficients were determined by measuring the Trp fluorescence and radioactivity or 12-AS fluorescence of the pellet and supernatant. Count rates of the pellet (C_P), supernatant (C_S), and the precentrifuged sample (C_T) were used to determine K_p according to

$$K_p = \frac{C_P}{C_S - (V_{ms}/V_m)(C_S + C_P)} \frac{V_S}{V_m} \tag{2}$$

in which V_{ms}/V_m is obtained from the ratio of supernatant to pellet + supernatant Trp fluorescence and corrects for the membrane remaining in the supernatant. A similar procedure

Table I: Amphipath Partition Measured by Hygroscopic Desorption[a]

amphipath	membrane	[total FA] (μM)	[membrane PL] (μM)	$C_F \times 10^{-3}$	$C_T \times 10^{-3}$	$K_P \times 10^{-3}$
PA	0	1.35	0	8.7 ± 0.2	13	
	PC	1.35	5	10.9 ± 0.5	16	2.3 ± 0.1
	CH1	1.35	5	16.4 ± 0.5	20	34 ± 1
PA	0	1.35	0	3.2 ± 0.2	28	
	PC	1.35	5	5 ± 2	31	9 ± 4
	CH1	1.35	5	6.4 ± 0.9	35	14 ± 2
PA	0	1.25	0	14 ± 1	66	
	PC	1.25	100	22 ± 2	68	1.2 ± 0.1
OA	0	1.35	0	2.8 ± 0.7	30	
	PC	1.35	5	4.2 ± 0.2	29	10 ± 2
	CH1	1.35	5	9 ± 2	35	37 ± 9
LA	0	1.35	0	3.0 ± 0.5	33	
	PC	1.35	5	5.0 ± 0.9	33	12 ± 2
	CH1	1.35	5	6 ± 1	34	18 ± 3
				I_f	I_t	
12-AS	0	5	0	61 ± 8	88	
	band 3 vesicles	5	90	79 ± 9	88	2.7 ± 0.2

[a] Abbreviations: PA, palmitic acid; OA, oleic acid; LA, linoleic acid; PC, egg phosphatidylcholine; PL, phospholipid; FA, fatty acid; C_F, cpm retained on the filter; C_T, cpm added to the filter (prefiltrate). C_T, C_F, and K_P values are the averages of three separate measurements, and the errors are standard deviations. I_f is the fluorescence intensity of the 12-AS (ethanol extracted) from the filter. I_t is the intensity of a 5 μM solution of 12-AS in ethanol.

in which the C's were replaced by the fluorescence intensities of the corresponding samples was used to determine K_p of 12-AS.

Fluorescence Titration. Fluorescence titrations were performed at room temperature by adding small volumes (1–5 μL) of a concentrated (2–35 mM) ethanolic solution of 12-AS to a suspension of red cell ghosts (50–200 μM phospholipid). The suspension was incubated for 15 min, and peak fluorescence intensities were evaluated at each addition after subtracting the contribution from a suspension without 12-AS. The partition coefficient was determined from the slope (α) of the fluorescence intensity [I(12-AS)] as a function of the total 12-AS concentration ([12-AS]) according to

$$I(\text{12-AS}) = C[\text{12-AS}]_m \quad (3)$$

in which C is given by

$$C = I^o(383)\epsilon(\text{12-AS})Q(\text{12-AS}) \quad (4)$$

where ϵ(12-AS) is the molar extinction coefficient of 12-AS at 383 nm and Q(12-AS) is the quantum yield. The quantity I^o(383) is proportional to the exciting light intensity at 383 nm and can be determined in terms of the intensity of the quinine sulfate (QS) standard according to

$$I(\text{QS}) = I^o(352)\epsilon(\text{QS})Q(\text{QS})[\text{QS}] \quad (5)$$

in which $I^o(383)/[I^o(352)] = 1.7$ and $Q(\text{QS})/[Q(\text{12-AS})] = 1.5$. In terms of eq 3–5, the partition coefficient can be expressed as

$$K_p = \alpha/(C - \alpha) \quad (6)$$

In deriving this expression, we have neglected the aqueous phase contribution of 12-AS fluorescence. This contribution is negligible since the quantum yield of 12-AS in membrane is 30 times greater than in water.

Results

Hygroscopic Desorption. Filtration under our conditions did not proceed entirely spontaneously, and it was necessary, therefore, to apply suction. The resistance to filtration was proportional to the membrane concentration, requiring 15 min for complete filtration with no membrane, 15 min with 5 μM phospholipid, and ≥30 min with 100 μM phospholipid (the maximum used). The reduction in flow rate did not, however, result in unacceptable levels of filter background. In all cases, less than 0.5% of the ^3H_2O or [^{3}H]inulin tracer was retained on the filter.

The results of measurements carried out with palmitic, stearic, oleic, and linoleic acids in egg phosphatidylcholine vesicles and CH1 plasma membranes are shown in Table I. In most of the measurements, less than 15% of the total amount of fatty acid added to the membranes was retained on the filter. This degree of retention was not affected by the method of filter treatment or by the type of fatty acid. Although the background was high, the amount of radioactivity observed in the presence of membranes was, with a single exception, about 50% greater than background. A Student's t-test analysis of most of the results in this table indicates that the probability of $K_p = 0$ is <0.02; in the worst two cases, p was <0.14 and 0.28, respectively. As shown in Table I, the apparent partition coefficients were similar in egg phosphatidylcholine vesicles and CH1 membranes and were in the range of 1×10^3 to 4×10^4.

Also shown in Table I are the results obtained by using the fluorescent fatty acid analogue 12-AS in red cell ghosts and band 3 vesicles. Although the 12-AS background levels are high, the amount of 12-AS retained on the filter was enhanced in the presence of membranes. The partition coefficient obtained from these studies is greater than 2×10^3 in ghosts and vesicles.

In order to compare our results with those of Conrad & Singer (1979, 1981), we measured the partition of chlorpromazine into CH1 plasma membranes. Much higher levels of chlorpromazine were retained on the filter in our measurements (Table II) than observed by Conrad and Singer. As seen in the table, our results are consistent with a large chlorpromazine partition coefficient ($>10^3$) in CH1 membranes.

Centrifugation. Centrifugation was used to determine the K_p of fatty acids in CH1, and platelet, membranes and the K_p of 12-AS in band 3 vesicles. As much as 40% of the total plasma membrane (presumably small vesicles or fragments) remained in the supernatant following centrifugation. Supernatant-associated membrane was accounted for by using Trp fluorescence. The correction for the supernatant contribution amounted to, at most, a factor of 2 increase in K_p.

Table II: Membrane/Water Partition of Chlorpromazine by the Hygroscopic Desorption Method[a]

amphipath	membrane	[total PF] (μM)	[membrane PL] (μM)	$C_F \times 10^{-3}$	$C_T \times 10^{-3}$	$K_P \times 10^{-3}$
chlorpromazine	0	1.3	0	600 ± 200	1500	6 ± 2
	0	1.3	0	170 ± 30	1500	70 ± 10
	CH1	1.3	5	800 ± 100	2000	
	0	1.3	0	80 ± 20	760	
	CH1	1.3	100	430 ± 30	800	7.3 ± 0.5
	0	1.3	0	92 ± 3	730	
	PC	1.3	100	110 ± 10	640	0.45 ± 0.05
	CH1	1.3	100	260 ± 20	710	3.0 ± 0.2

[a] Parameters and experimental conditions are the same as those in Table I.

Table III: Free Fatty Acid Partition by Centrifugation[a]

amphipath	membrane	[total FA] (μM)	[membrane PL] (μM)	C_T	C_P	C_S	V_{ms}/V_m	$K_P \times 10^{-3}$
SA	platelet	12.5	50		140	20	0.11	110 ± 20
	platelet	12.5	50	890	660	150	0.17	100 ± 20
	CH1	37.5	500	830	730	90	0.10	17.4 ± 0.7
	CH1	12.5	50	1470	970	140	0.09	46 ± 3
OA	platelet	12.5	50		270	100	0.25	69 ± 7
	platelet	12.5	50	540	300	270	0.46	73 ± 0.4
	CH1	37.5	500	690	480	130	0.17	3.5 ± 0.4
	CH1	12.5	50	580	630	150	0.05	11 ± 1
LA	CH1	12.5	50	980	580	690	0.14	2.1 ± 0.1
				I_t	I_p	I_s		
12-AS	band 3	2.2	200	98	76	22	0.22	800 ± 80

[a] Abbreviations: C_T, total cpm; C_P, pellet cpm; C_S, supernatant cpm; V_{ms}, lipid volume of the supernatant; V_m, total lipid volume. I_t is the fluorescence intensity of 12-AS in band 3 vesicles before centrifugation, I_p is the intensity of the pellet, and $I_s = I_t - I_p$.

The results of the partition coefficient determination using centrifugation are shown in Table III and demonstrate that in all cases K_p is greater than 10^3.

Fluorescence Titration. The fluorescence titration method was used to determine the membrane/water partition coefficient of 12-AS in red cell ghosts. The results of this study are shown in Figure 1, where it is seen that the intensity vs. total [12-AS] is linear for [phoshpholipid]/[12-AS] > 50/1. A least-squares fit to the intensities in the linear region, together with eq 3–6, yields a K_p value $>10^2$.

Discussion

We have investigated the membrane/buffer partition behavior of fatty acids and a fluorescent analogue in plasma membranes of red cells, lymphoma, and platelets and in artificial egg phosphatidylcholine membranes. The following three different approaches were used: hygroscopic desorption, centrifugation, and fluorescence titration. No significant partition difference was observed between artificial and plasma membranes. All three methods for all fatty acid/membrane combinations yielded partition coefficients in the range of 10^2–10^5. These results suggest, therefore, that fatty acids can readily partition into artificial and cell plasma membranes.

In the present study, we did not determine the nature of this partition. There is, however, direct evidence for fatty acid partitioning into the hydrophobic region of whole red cell membranes from energy transfer measurements between hemoglobin and the *n*-(9-anthroyloxy) (AO) fatty acids (Shaklai et al., 1977; Eisinger & Flores, 1982). In these studies, the AO probes were added to the outside of whole red cells, and it was found that the hemoglobin/anthroyloxy separations were less than 45 Å (44 Å for 2-AS and 30 Å for 16-AS). Such short separations are incompatible with adsorption of the fatty acids in the form of micelles or hemicapped micelles to the surface of the cell. Direct evidence for the location of the AO moiety in the hydrophobic region of

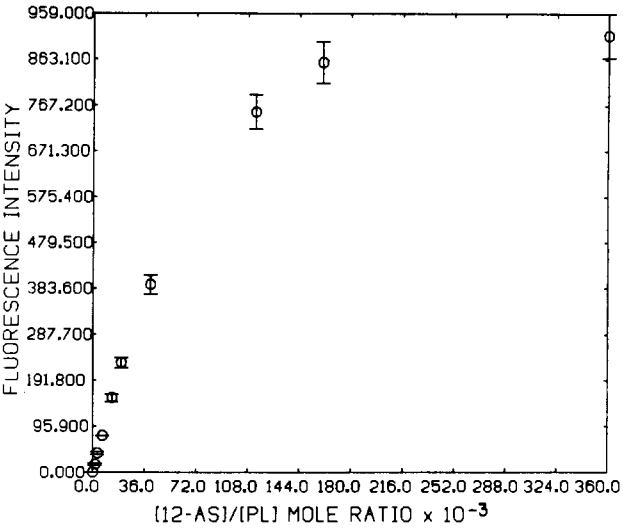

FIGURE 1: Titration of 12-AS and ghosts. 12-AS fluorescence intensity was measured as a function of the mole ratio of [12-AS]/[phospholipid]. The emission intensity of 12-AS is in arbitrary units. The phospholipid (PL) concentration of the red cell ghosts used in this measurement was 200 μM. The partition coefficient was determined from the linear region of the titration curve at PL/probe ratios of less than 50/1.

lipid bilayers is provided by X-ray diffraction (Lesslauer et al., 1972) and by proton NMR (Podo & Blaisie, 1977). In the X-ray diffraction studies, the major effect of 12-AS in dipalmitoylphosphatidylcholine was a broadening of the electron density profile in the region of the methyl trough, as expected for an AO moiety located near the center of the bilayer. The NMR study demonstrates that only the resonances corresponding to the $(CH_2)_{4-15}$ protons are chemically shifted by 12-AS incorporation. 12-AS also altered the T_1 relaxation rate only of the terminal methyl group. The AO moiety must, therefore, be located in the hydrocarbon region

since these effects are due to short-range interactions between protons and the AO ring system.

Additional evidence for the buried location of the AO moiety is provided by our own studies of the quenching of AO fluorescence (Chalpin & Kleinfeld, 1983). We demonstrated that in the presence of red cell ghosts or artificial phosphatidylcholine liposomes, the long-chain AO fatty acids are inaccessible to aqueous phase quenchers of fluorescence such as acrylamide. Results obtained with KI demonstrated that, in membranes, the depth of the AO moiety corresponds to its position on the fatty acid acyl chain. Most important, the quenching behavior was virtually identical in ghosts and artificial liposomes. On the other hand, the quenching behavior, of either acrylamide or KI, in aqueous micelles of AO bore no relationship to the results obtained in the presence of membranes. The same conclusions have been reached in studies using spin-labeled fatty acids and a dansylated propranolol (Moules et al., 1982). These studies demonstrate that the spin-labeled and fluorescent probes partition similarly into the lipid phase of dioleoylphosphatidylcholine liposomes and membranes derived from sarcoplasmic reticulum.

We also carried out hygroscopic desorption measurements with chlorpromazine, one of the amphipaths studied by Conrad & Singer (1981). Our findings revealed no difference between plasma membranes and artificial liposomes. The K_p values we obtain [$(1 - 10) \times 10^3$] are consistent with those of Roth & Seaman (1972) and are inconsistent with the value of zero found by Conrad & Singer (1981). Bondy & Remien (1981) reached a similar conclusion. These authors used the hygroscopic desorption method to measure the partition of chlorpromazine into whole cells (erythrocytes, platelets, lymphocytes, granulocytes) and red cell ghosts. The salient feature of their study was that K_p ranged between 40 and 3500, depending upon cell type and pH, for chlorpromazine concentrations less than 60 μM. Above this concentration, however, they observed a percipitous decrease in K_p, which they ascribed to membrane damage. Since the concentration of chlorpromazine in our measurements was 1.3 μM, it is likely that the high concentration (60 μM) used by Conrad & Singer (1981) is the source of discrepancy in the various studies.

Although we observed considerable fluctuations in K_p [probably because of the higher backgrounds in our measurements than in those of Conrad & Singer (1981) or Bondy & Remien (1981)], in no single measurement did we obtain a value for K_p which was less than 10^2 nor did we observe any significant difference between lipid bilayers and membranes. Taken together with the above-mentioned studies which demonstrate a hydrophobic location of fatty acid analogues, we conclude, in contrast to Conrad & Singer (1981), that fatty acids can partition into the hydrophobic region of plasma membranes. The work of Conrad & Singer (1981) does, however, raise the possibility that some amphipaths may preferentially partition into the interface region between the bulk aqueous phase and the hydrocarbon region of the membrane. We emphasize this issue since our own work (Chalpin & Kleinfeld, 1983) indicates that short-chain fatty acids, in this case 11-AO-undecanoic acid, bind to the surface but do not penetrate into the interior of red cell ghosts. In addition, Bondy & Remien (1981) suggest that chlorpromazine also binds to a superficial region of membranes.

Registry No. PA, 57-10-3; OA, 112-80-1; LA, 60-33-3; 12-AS, 30536-60-8; SA, 57-11-4; chlorpromazine, 50-53-3.

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