ELSEVIER

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Interaction of *N*-(2-hydroxybenzyl)-ω-amino carbonic acids, novel amphipathic fatty acid derivatives, with membrane: partition coefficients

Elena Lurie *, Alexander Kaplun, Ivan Vassilenko, Peter Dubovskii, Vitaly Shvets

Biotechnology Department, M.V. Lomonosov Institute of Fine Chemical Technology, B-571 Prospect Vernadskogo 86, 117571 Moscow, Russian Federation

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Abstract

The methods for partition coefficient (K_p) determination were developed for different concentrations of *N*-(2-hydroxybenzyl)-ω-amino carbonic acids, a new class of amphipathic fatty acid derivatives (A*n*), their deutero (A*n*D) and bromine (A*n*Br) derivatives. To do this the following methods were used: ^{2}H-NMR, equilibrium dialysis, centrifugation and fluorescence spectroscopy. K_p dependence on the A*n* concentration is discussed. K_p values for A*n*Br were more than 120-times higher than those for A*n*, the differences between them being smaller than those for the corresponding A*n*. This series of new amphipathic compounds can be used as probes for membrane studies.

Keywords: Amphipathic fatty acid derivative; Fatty acid; Membrane; Partition coefficient; NMR, ^{2}H-; Equilibrium dialysis; Fluorescence spectroscopy

1. Introduction

Many molecules of biological interest have amphipathic character [1,2]. Understanding of the interaction between amphipathic compounds and a membrane could be very helpful for a molecular level analysis of such phenomena as drug-membrane interaction, membrane solubilization [3], and others. The effect produced by the compound on membrane components depends on the compound concentration in a membrane. The quantity of the compound distribution between the lipid and the aqueous phases is expressed by an equilibrium partition coefficient (K_p).

Here, we give partition coefficients for a novel class of amphipathic fatty acid derivatives, *N*-(2-hydroxybenzyl)-ω-amino carbonic acids (A*n*) (Fig. 1). The aryl substituted fatty acids have taken attention of biochemists since the classic works of Knoop [4] as useful tools for investigation of fatty acid metabolism [5], as fluorescence and photoactivated probes for membrane investigations [6], and as diagnostic preparations [7–10]. With the help of An^{125}I it was shown that A*n* underwent β-oxidation in vivo [11]. So the acids with long chain and even number of C-atoms were converted into acids with shorter chain length, mostly into A4^{125}I, and the acids with the odd number of C-atoms – into A3^{125}I. The fact that A*n* can act as substrate of the β-oxidation enzymes was proved by their inhibition of β-oxidation of natural fatty acids ([1-^{14}C]oleic acid and [1-^{14}C]stearic acid). A*n* can essentially broaden the use of F. Knoop fatty acid derivatives because they can be labelled with radioactive isotopes, which correspondingly will increase the sensitivity of the method.

The first step in the study of interaction between A*n* and PC membrane deal with the investigation of their influence on the membrane structure. The ^{31}P-NMR spectra of PC/A*n* membrane at PC/A*n* ratio from 20:1 to 5:1 were close to the theoretical calculated one-component spectrum in the temperature range of 27–55° C [12]. The theoretical spectrum major components of the chemical shift anisotropy tensor of phosphorus nucleus (S_{ii}, $i = 1, 2, 3$) were calculated and the chemical shift anisotropy parameter $\Delta\sigma$ ($\Delta\sigma = (S_{11} - 1)/2(S_{22} + S_{33})$) was close to −50 ppm. According to the literature data [13,14] this value of $\Delta\sigma$ corresponds to bilayer structure of PC mem-

Abbreviations: A*n*, *N*-(2-hydroxybenzyl)-ω-amino carbonic acids, where *n* is a number of C-atoms in the chain; A*n*D and A*n*Br, corresponding derivatives with deuterium and bromine in the aromatic ring; K_p, partition coefficient; PC, phosphatidylcholine; SUVs, small unilamellar vesicles; LMVs, large multilamellar vesicles.

* Corresponding author. Fax: +7 095 4375500.

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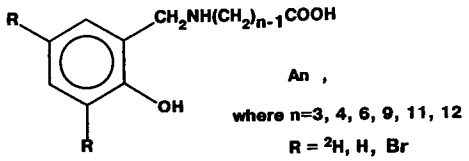

Fig. 1. Structure of *N*-(2-hydroxybenzyl)-ω-amino carbonic acids and their derivatives.

brane. So the results obtained showed that A*n* do not disturb the membrane bilayer structure.

Thanks to the compound molecule structure it is possible to use several methods for K_p determination. The phenyl ring absorption in the UV light makes it is possible to use spectrophotometrical methods. Also the phenyl ring at the end of the chain allows a rather easy introduction of deuterium (^{2}H-NMR studies) and bromine (fluorescence spectroscopy). Bromine introduction into A*n* gives derivatives that are fluorescence quenchers [15]. Therefore K_p for these compounds can be determined by fluorescence quenching of the fluorescent membrane probe [16,17].

2. Methods

2.1. Materials

The egg PC was purchased from Kharkov Plant 'Biolek' (Ukraine). Pyrene-labelled PC (pyrene moiety in hydrophobic region) was obtained as described in [6].

A*n* and A*n*Br were synthesised as described in [11], except that $NaBH_4$ was added in 4 ml of methanol. A*n*D were synthesised as described in [12].

2.2. Sample preparation

In the ^{2}H-NMR and centrifugal experiments LMV's containing A*n*D (A*n*) were prepared in the following way. Methanolic solutions of A*n*D (A*n*) and PC were mixed in 1:5 (mol/mol) ratio. The obtained solution was evaporated in a 50 ml round bottom flask under nitrogen, and the film was dried in a vacuum desiccator over P_2O_5 for 24 h. The dried residue was supplemented with 0.05 M Tris-HCl buffer (pH 7.2) to give samples for ^{2}H-NMR studies with PC/H_2O ratio (mol/mol) 1:200 and for the centrifugal studies with 1:250 and 1:900 ratios. Then the samples were vortexed for 15 min. In order to attain equilibrium of the A*n*D (A*n*), each sample was subjected to five freeze-thaw cycles. These cycles took 2 h, the freeze temperature was −54° C, and the thawed temperature was 45° C. Spectra recorded immediately after freeze-thaw treatment were identical to those made 24 h later, thus showing that the system was in equilibrium. The freeze-thaw equilibrated samples were kept at the room temperature during this time.

SUV's for equilibrium dialysis and centrifugation were prepared by established procedure [18]. The methanolic solution of PC was injected through a small-bore needle into a rapidly stirred 0.05 M Tris-HCl buffer (pH 7.2). The final PC concentration in the buffer was 1.66 mg/ml. PC dispersion was stirred for 40 min and then sonicated eight times (0.5 × 1 min) under nitrogen at 4° C and 44 kHz using an UZDN-2T ultrasonic sonicator (Russia).

2.3. Deuterium NMR

Spectra were recorded on a MSL-200 spectrometer (Brucker, Germany) at a deuterium frequency of 30.72 MHz. Spectra were acquired by using the quadruple echo pulse sequence [19,20] with full phase cycling of the radio-frequency pulses. Pulse spacing was 50 μs, the $\pi/2$ pulse length was 8.5 μs. The typical acquisition parameters were: the spectral width – 150 kHz, the number of data points – 2K, relaxation delay – 0.5 s, number of scans – 100 000. Before Fourier transformation the echo signals were exponentially multiplied with line-broadening factors being in the range 30–100 Hz. This data treatment did not cause additional line-broadening and did not influence simulation results of the experimental spectra. The simulated spectra were calculated using the Pascal-written programs run on the computer Aspect 3000 of MSL 200 instrument. The anisotropic components were calculated as superposition of two Pake doublets with Gaussian line shape. The isotropic components were approximated by single Lorentzian lines. The simulated spectra obtained in such way corresponded to the experimental spectra much better than with other combination of line types for isotropic and anisotropic signals. The estimated inaccuracies in the line-width determination were well within 10% of reported δ values.

To determine the aqueous phase HDO contribution due to the natural abundance of HDO in H_2O to the intensity of the isotropic signal, the spectra of the control samples without A*n*D were recorded at the same conditions as the investigated samples. The areas of single isotropic signals [21] were subtracted from isotropic signals of experimental spectra (A*n*D/PC/H_2O). After this procedure the corrected areas of isotropic signals were used to give K_p values in the same manner as in the work [3].

2.4. Centrifugation

The centrifugation was carried out on Beckman L7-55 (USA) centrifuge, rotor SW-65. The samples containing LMV's and A*n* with PC/H_2O rations (mol/mol) 1:250 and 1:900 were centrifuged at 300 000 × *g* for 40 min, and the supernatants were removed and diluted with 0.05 M Tris-HCl buffer (pH 7.2). Spectrophotometric measurements of A*n* concentration were taken with a spectrophotometer Shimadzu UV-240 (Japan) at 275 nm. TLC of

the supernatant in $CHCl_3$-MeOH-H_2O (65:25:4) mixture did not show any traces of PC.

The aliquots of $2.4 \cdot 10^{-2}$ M methanolic solutions of A4 (A6 or A9) or/and $8 \cdot 10^{-3}$ M methanolic solution of A11 (A12) were added to SUV's. The final A*n* concentration in the dispersion was $2.4 \cdot 10^{-4}$ M. The samples containing SUV's and A*n* were centrifuged at $300\,000 \times g$ for 2 h. The supernatants were contaminated by lipids according to TLC data. The absorption of the samples at 275 nm was higher than the absorption of the control samples ($2.4 \cdot 10^{-4}$ M A*n* solution in 0.05 M Tris-HCl buffer (pH 7.2)).

2.5. Equilibrium dialysis

2 ml of egg PC SUV's dispersion was placed in 1 cm diam visking dialysis tubing bags. The bags were placed into 8 ml 0.05 M Tris-HCl buffer (pH 7.2). The aliquots of $2.4 \cdot 10^{-2}$ M methanolic solutions of A4 (A6 or A9) and $8 \cdot 10^{-3}$ M methanolic solution of A11 (A12) were added to the external buffer solution. The final A*n* concentration was $2.4 \cdot 10^{-4}$ M. In a parallel set of experiments A4 (A6 or A9) was added to the dialysis bags containing the SUV's. The dialysis was performed at 20° C. The external buffer was continuously stirred by magnetic stirrers. The incubation was terminated when the A*n* concentration reached a plateau as found from its absorption at 275 nm. In all cases 4 h were sufficient for equilibration. The volume of the lipid phase was calculated as described in [22].

2.6. Fluorescence quenching

The liposomes were prepared using pyrene-labelled PC (3 mol%) in the same way as for the equilibrium dialysis. The PC concentration in the buffer was 0.05, 0.1, 0.15 or/and 0.2 mg/ml. The 40 μl of A*n*Br solution in methanol was added to the SUV, the control samples containing 40 μl of methanol only. The A*n*Br concentration varied from 10^{-5} M to 10^{-3} M. Fluorescence was measured on Hitachi F-4000 (Japan) and Hitachi 650-60 (Japan) spectrofluorimeters. The excitation wavelength was 337 nm and the emission wavelength was 376 nm.

3. Results and discussion

Equilibrium partition coefficient is a ratio of the concentration of the compound dissolved in the lipid phase to that in the aqueous phase at equilibrium [23]. K_p can be calculated using Eq. (1):

$$K_p = \frac{[Q]_l}{[Q]_a} = \frac{M_l V_a}{M_a V_l} \quad (1)$$

where $[Q]_l$ and $[Q]_a$ are the compound concentrations in the lipid and aqueous phases, respectively; V_l is the volume of the lipid phase; V_a is the volume of the aqueous phase; M_l is the amount of the compound in the lipid phase; and M_a is the amount of the compound in the aqueous phase.

According to Eq. (1) we see that to calculate K_p it is necessary to measure the compound concentration in the aqueous and/or the lipid phase(s). This was done by UV-spectroscopy, ^{2}H-NMR and fluorescence spectroscopy.

Since UV-spectroscopic parameters for A*n* from the aqueous and the lipid phases are very similar it is not possible to isolate signals from two phases. Therefore we have to separate the phases, which can be done by centrifugation [24], or by equilibrium dialysis [22]. In separated phases we already can do concentration measurements.

The different spectral properties of the compounds in the aqueous and the lipid phases allows to make measurements without separating the two phases. The ^{2}H-NMR spectra observed for the deuterated A*n* species in PC dispersions are characterised by the presence of a wide anisotropic resonance and a sharp isotropic resonance (Fig. 2). The area of the isotropic resonance corresponds to the A*n*D amount in the aqueous phase, and the area of the anisotropic resonance – to the A*n*D amount in the lipid phase. One of the drawbacks of the ^{2}H-NMR method is low sensitivity, and therefore we have to use high concentrations of the compound. K_p in this case can be in the concentration-dependent area. But these particular K_p values can be useful for the data interpretation in ^{2}H-NMR experiments, because most of such experiments are carried out using the high compound concentrations.

The K_p values were calculated from the ^{2}H-NMR spectra: the ratio of the anisotropic and isotropic resonance areas were divided by the ratio of the lipid and the aqueous phase volumes. For model membranes we have used liposomes prepared by hydration of the PC/A*n*D films with aliquots of 0.05 M Tris-HCl buffer (pH 7.2). In order to attain complete equilibration of A*n*D, each sample was subjected to five freeze-thaw cycles [24]. The equilibrated state of the samples was shown by comparison of their spectra with those obtained 24 h later. The PC/A*n*D ratio was 5:1 (mol), and the A*n*D concentration was $5.56 \cdot 10^{-2}$ M. The K_p values are presented in Table 1.

As a further check, the free A*n* concentration of the ^{2}H-NMR samples was determined by the centrifugation method in the very similar conditions to ^{2}H-NMR method. The PC/H_2O ratio (mol/mol) was 1:250, and A*n* concentration was $4.4 \cdot 10^{-2}$ M. The K_p values were considerably larger than those observed for the ^{2}H-NMR samples. The difference could be due to the three states of A*n* in the aqueous-lipid system: A*n* molecules in the aqueous phase, A*n* molecules weakly bound to PC membrane, and A*n* molecules strongly bound to PC membrane. The similar scheme was proposed for the interaction of local anesthetics with PC membrane [23]. In the case of ^{2}H-NMR

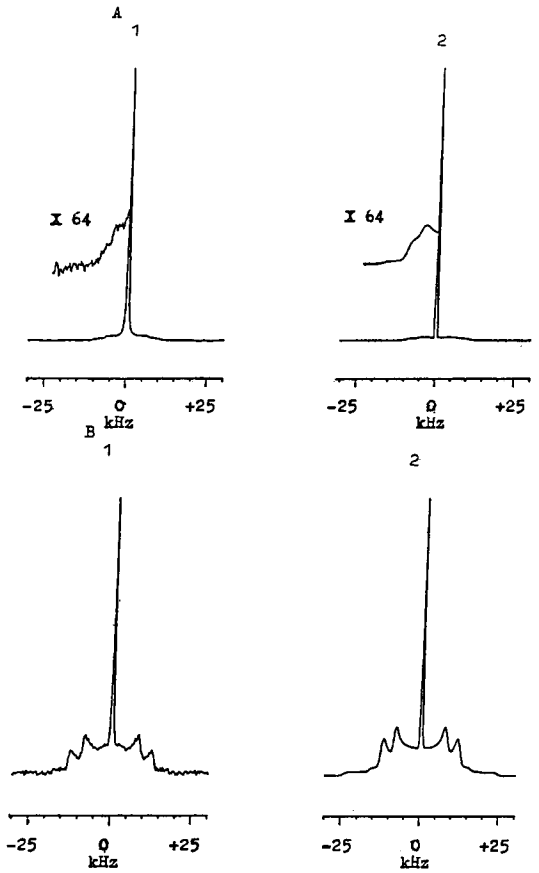

Fig. 2. ^{2}H-NMR spectra of PC/A4D (A) and PC/A12D (B) at 40° C: 1, experimental spectra; 2, simulated spectra, where isotropic component was calculated as Lorentzian line with half-height linewidth of 550 Hz; anisotropic component was calculated as superposition of two Pake doublets with Gaussian line-shape. Quadrupole splitting ($\Delta\nu$, kHz) and half-height linewidth ($\Delta\nu_a$, kHz) for theoretical spectra were following: A, $\Delta\nu(1) = 1600$, $\Delta\nu_a(1) = 18.0$, $\Delta\nu(2) = 1600$, $\Delta\nu_a(2) = 10.0$; B, $\Delta\nu(1) = 750$, $\Delta\nu_a(1) = 24.5$, $\Delta\nu(2) = 750$, $\Delta\nu_a(2) = 16.3$. The anisotropic component at A spectra is given ×64-fold.

method the weakly bound AnD contributed to the isotropic signal, and thus they corresponded to the aqueous phase. In the centrifugal method these molecules were in the pellet. Therefore, K_p values determined in the ^{2}H-NMR method were lower than those obtained in the centrifugation.

K_p at lower An concentrations was studied by centrifugal method. The samples were prepared as described above. The PC/An ratio was 5:1 (mol), and the An concentration was $1.23 \cdot 10^{-2}$ M. The concentration of An in the supernatant was determined spectrophotometrically. The An amount in the lipid phase was calculated by subtraction of the An amount contained in the aqueous phase from the total amount of the compound. There were no significant differences between K_p values determined for the different conditions (PC/H_2O ratio (mol/mol) 1:250, An concentration $4.4 \cdot 10^{-2}$ M, and PC/H_2O ratio (mol/mol) 1:900, An concentration $1.23 \cdot 10^{-2}$ M) using the centrifugal method.

Determination of K_p values in more diluted systems can be done using equilibrium dialysis [22]. K_p determination via equilibrium dialysis does not require complete phase separation which, of course is a great merit of the method. The liposomes were prepared by injection of methanolic PC solution into 0.05 M Tris-HCl buffer (pH 7.2) in such concentration that the methanol content in the final dispersion did not exceed 1%. The dispersion obtained was consequently sonicated. Of course, it is generally undesirable to change the preparative techniques, but this was caused by the following reasons. Unilamellar vesicles are more suitable for equilibrium dialysis because they have fewer separate aqueous and lipid compartments than multilamellar vesicles. This affects the time needed for attaining the equilibrium. Multilamellar vesicles require much more time for equilibration as An molecules have to penetrate the outer membrane layers. Also vesicles do not precipitate during the experiment because of their small size. These are the reasons why SUV's are more convenient for equilibrium dialysis. However in the previous cases (^{2}H-NMR and the centrifugation) LMV's are preferable. SUV's have narrow isotropic signal in NMR spectra due to lateral lipids diffusion and vesicles rotation. The main data in our case are line-shape and quadrupole splitting [19], and using SUV's we loose this information. Therefore it is not feasible to use them for ^{2}H-NMR

Table 1
Partition coefficients of An

An	^{2}H-NMR spectroscopy (H_2O/PC, 200:1, mol/mol) AnD $5.56 \cdot 10^{-2}$ M		Centrifugation (H_2O/PC, 250:1, mol/mol) An $4.4 \cdot 10^{-2}$ M		Centrifugation (H_2O/PC, 900:1, mol/mol) An $1.23 \cdot 10^{-2}$ M		Equilibrium dialysis (H_2O/PC, 8800:1, mol/mol) An $2.4 \cdot 10^{-4}$ M	
	K_p	PC/An mol/mol in membrane	K_p	PC/An mol/mol in membrane	K_p	PC/An mol/mol in membrane	K_p	PC/An mol/mol in membrane
A4	1.4	19	1.8	19	2.1	53	24	256
A6	2.4	13	7.6	15	9.5	16	25	247
A9	15.7	6	52	6	57	7	60	118
A11	17.0	5	130	5	136	5	144	65
A12	17.5	5	207	5	250	5	255	48

experiments. In centrifugation LMV's precipitate at lower acceleration (g) than SUV's. Therefore ^{2}H-NMR and centrifugation experiments were carried out using LMV's.

The compound under investigation was added to the external buffer solution, and in the parallel set of experiments to the dialysis bag containing SUV's. PC/An ratio was 9.1:1 (mol), and the An concentration was $2.4 \cdot 10^{-4}$ M. In all cases, 4 h were enough for system to equilibrate as was indicated by constant An concentration in the external buffer solution. The final An concentration in the external buffer was not affected by the An addition technique. The initial data for K_p calculation were the An concentration in the external buffer solution equal to the An concentration in the aqueous phase. Knowing the volumes of the aqueous and lipid phases and the total amount of An, we could calculate the concentration of An in the lipid phase.

With equilibrium dialysis we cannot obtain such a high concentration as with ^{2}H-NMR and centrifugation for two reasons. First of all An have low solubility, so that we cannot dissolve the required amount of the compound in the required amount of the solvent. Secondly, the external phase volume must be big enough for the dialysis bag to be fully immersed in it.

In order to compare results obtained we carried out experiments, where K_p values were determined by the centrifugal method under the equilibrium dialysis condition. However, after SUV's centrifugation (300 000 × g, 2 h) there were always some lipids in the supernatant, and even in the control samples (pure PC vesicles without An). The destruction of the small vesicles in the supernatant using deoxycholate did not bring positive results. So, we cannot estimate the An concentration in the aqueous phase, because the PC molecules contributed the absorption at 275 nm.

Determination of partition coefficients is quite a complicated problem. The inconclusive results obtained are due to the incompatibility between the realistic system to the ideal one where every phase is defined as "the part of the system which is chemically and physically uniform throughout" [1].

Data given in Table 1 and Fig. 3 are in agreement with the general rule that An affinity to egg PC liposomes increased with higher hydrophobicity of An.

For halogen derivatives of An that are fluorescence quenchers [15] K_p can be found from the efficacy of the fluorescence quenching. This method is based on the Encinas and Lisso technique [1] which main assumption is that "I_0/I at a particular quencher concentration will only be dependent on the average number of quencher per lipid structures, and will be independent of the quenching mechanism, the quenching efficiency or the statistical distribution of the quencher between the lipid structures" [1]. Since the quenching of the hydrophobic fluorescence probe depends on the ability of a quencher to penetrate into the lipid phase [13], therefore K_p for AnBr, the fluorescence quenchers (Fig. 4a), can be determined from the fluorescence quenching of the pyrene-labelled PC. Quenching dynamics for a fluorophore in solution is described by the Stern-Volmer equation [25]:

$$\frac{I_0}{I} - 1 = k_q \tau [Q]_t \qquad (2)$$

where I and I_0 are the fluorescence intensities in the presence and the absence of quencher; k_q is the bimolecular rate constant, τ is the fluorescence lifetime, and $[Q]_t$ is

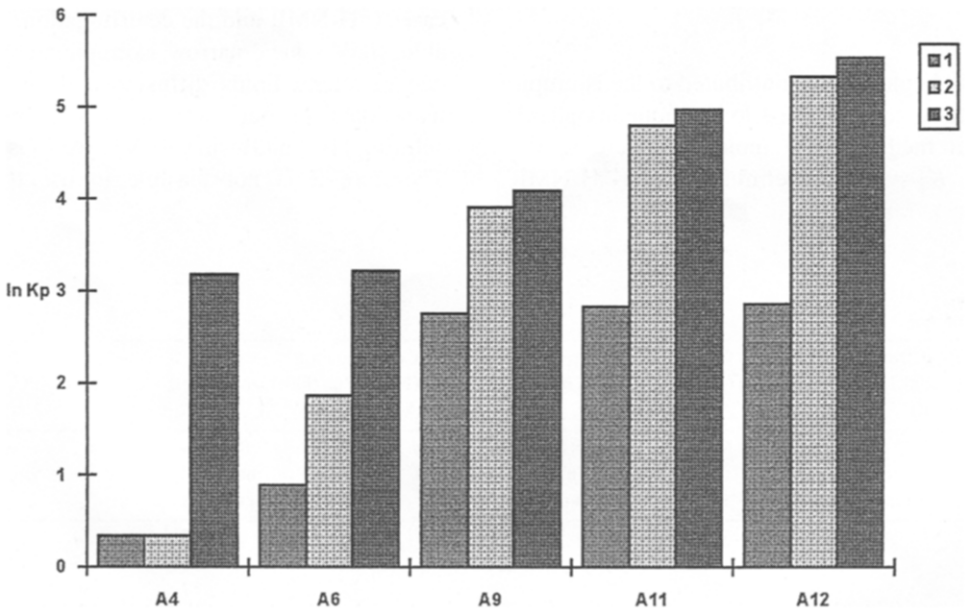

Fig. 3. Partition coefficients (K_p) of An (n = 4, 6, 9, 11, and 12): 1, ^{2}H-NMR experiments (H_2O/PC/AnD, 1000:5:1, mol/mol); 2, the centrifugation (H_2O/PC/An, 4500:5:1, mol/mol); and 3, the equilibrium dialysis (H_2O/PC/An 82 500:9:1, mol/mol).

the total concentration of quencher. For a quenching molecule which partitions into the lipid phase, the value of $[Q]_l$ may be taken, to a first approximation, as the concentration of the quencher in the lipid phase [16]. Combining Eqs. (1) and (2) gives:

$$\frac{I_o}{I} - 1 = k_q \tau K_p [Q]_a \quad (3)$$

The total amount of quencher is described by Eq. (4):

$$V_t[Q]_t = V_a[Q]_a + V_l[Q]_l \quad (4)$$

In most experimental situations the volume of the lipid phase is small compared to the total volume $V_t = V_a + V_l \sim V_a$ thus combining Eqs. (1) and (4) we obtain Eq. (5). Using Eq. (3) we transform Eqn. (5) to give Eq. (6):

$$[Q]_a = \frac{[Q]_t V_t}{V_t + K_p V_l} \quad (5)$$

$$\frac{I_o}{I} - 1 = k_q \tau K_p \frac{V_t}{V_t + K_p V_l} [Q]_t \quad (6)$$

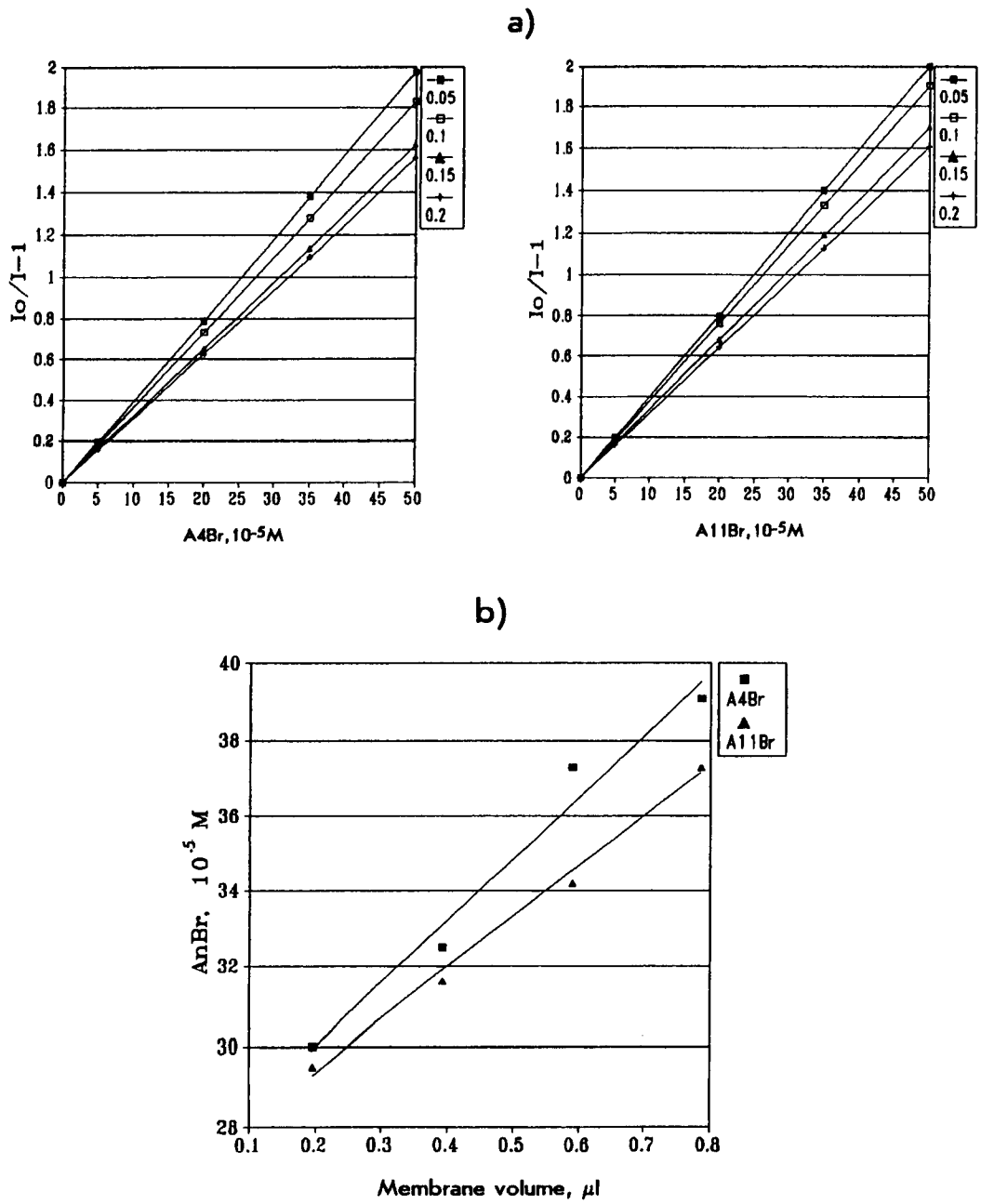

Fig. 4. Fluorescence quenching of the pyrene labelled PC with AnBr (λ_{ex} 337 nm, λ_{em} 376 nm): (a) the Stern-Volmer plots for AnBr at different concentrations of pyrene labelled PC: 0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml, and 0.2 mg/ml; (b) A4Br, and A11Br concentrations as functions of membrane volume at $(I_o/I) - 1 = \text{const}$.

The K_p values can be calculated by two ways. The first is like this: Eq. (6) gives $((I_0/I) - 1)$ versus $[Q]_t$ at constant V_l and V_a (Fig. 4a) and the lower the PC concentration the greater is the slope α:

$$\alpha = \frac{k_q \tau K_p}{V_t + K_p V_l} \quad (7)$$

Eq. (7) can be converted into:

$$\frac{1}{\alpha} = \frac{V_t}{k_q \tau K_p} + \frac{V_l}{k_q \tau} \quad (8)$$

The plot of $1/\alpha$ versus V_l yields a straight line [17] and K_p can be obtained from the slope and the intercept as follows:

$$K_p = \frac{\text{slope}}{\text{intercept}} V_a \quad (9)$$

The second way is based on the assumption, that the identical quenchers effects are brought about by the identical quencher concentrations in the membrane; therefore if we draw a straight line at a certain $((I_0/I) - 1)$ we shall obtain a series of points corresponding to identical $[Q]_l$. Now using the data obtained in the coordinate system $[Q]_t$ versus V_l we get a straight line (Fig. 4b), and from the slope and intercept of which we can obtain K_p by the procedure described above.

Hydrophobicity of A*n*Br is more affected by two bromine molecules than by the chain length of A*n*. Accordingly to this we decided to determine K_p for two boarded members of A*n* homologies line – A4Br and A11Br. K_p A4Br and A11Br were 33500 and 41400, respectively. A*n*Br have K_p values which are several orders higher than for A*n*, this is in conformity with the literature data [26]. The difference between the K_p values with a chain length increase is less for A*n*Br than A*n*. The other dependencies are similar to the described one.

High K_p values for A*n*Br make it possible to use them as membrane probes for fluorescence studies. ^{31}P-NMR experiments [12] showed that A*n*D do not disturb the bilayer structure of PC membrane at the PC/A*n*D ratio (mol/mol) 5:1, and using K_p obtained in ^{2}H-NMR we can calculate the A*n*D concentration in the lipid bilayer. So, A*n*D do not disturb the bilayer structure of PC membrane up to 15 M concentration in lipid phase. Thus deuterium derivatives of A*n* can be used as probes for ^{2}H-NMR studies of membranes. The simplicity of the synthesis and the possibility of introducing different labels into A*n* molecules make this novel class of amphipathic fatty acid derivatives a promising tool for biochemical and biophysical investigations of membrane processes.

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