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EVALUATION OF THE ANESTHETIC-LIPID ASSOCIATION CONSTANT

A MONOLAYER APPROACH

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A new approach is presented which allows to describe the binding of different local anesthetics to lipids. Lipids (DL-α-dipalmitoylphosphatidylcholine, phosphatidylserine, cardiolipin) are spread at the air-water interface and the anesthetic (procaine, butacaine, tetracaine) injected into the aqueous subphase. The equilibrium constants associated to the interfacial reaction:

$$D^+ \text{ (subphase)} + L^- \text{ (monolayer)} \rightleftharpoons DL \text{ (monolayer)}$$

(where D^+ denotes the anesthetics, L^- the lipid anionic site and DL the complex) are calculated from an experimental evaluation of the surface potential of the lipid monolayer. This mode of determination is based essentially on the good correlation between the experimental values of the surface potential and the theoretical predictions from the Gouy-Chapman theory. Fluorescence measurements on liposomes are carried out in order to locate the position of the drug in the lipid layer. This method can be extended to any positively charged drug-anionic lipid interaction.

Introduction

Most drugs must cross biological membranes prior to reaching their target. Therefore, a clear understanding of the mechanism of the drug-membrane interaction seems essential. However, the membrane composition is too complex to allow a simple analysis. A possible way to get an insight into this problem is to use model membranes which allow more specific testing of the lipid contribution in the drug-membrane interaction.

McLaughlin and Harary [1] showed that the Gouy-Chapman theory may be used to describe the binding of charged molecules to planar lipid bilayers. Lee [2] used the modification of lipid phase temperature induced by charged and uncharged drugs to calculate a drug-lipid dissociation constant. In this work, we present another approach allowing the evaluation of the drug-lipid association constant. The lipid is spread at the air-water interface and the drug injected into the aqueous phase [3–8]. Surface potential measurements obtained before and after complexation give direct information about the surface charge density. The agreement between the experimental values of the surface potential and the predictions of the Gouy-Chapman theory allows an immediate determination of the lipid-drug association constant. Fluorescence spectra of the drug in the presence of liposomes allow a qualitative evaluation of the degree of penetration of the drug into the lipid layer. The present paper describes the results obtained with the lipid-local anesthetic system. This system has been extensively studied [9–16] and for this reason was chosen to test the

validity of our approach. The role of the charge of the lipids and of the drugs structure (procaine, butacaine, tetracaine) was investigated in detail.

Material and Methods

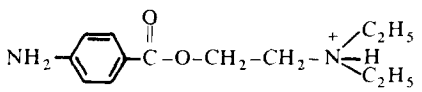
DL-α-Dipalmitoylphosphatidylcholine, cardiolipin, procaine, tetracaine and butacaine (Table I) were purchased from Sigma Chemical Co., and phosphatidylserine from Koch-Light Laboratory. Phospholipids were spread at the air-water interface from a chloroform solution using an Agla microsyringe. Water was triple distilled in presence of potassium permanganate. All experiments were carried out at 25°C. The aqueous subphase was an acetic acid–sodium acetate buffer (pH = 6.6; 10^{-1} or 10^{-2} M NaCl). Multilamellar liposomes were obtained by mechanical stirring (Vortex mixer) of a lipid film in buffer (Tris-HCl, 0.1 M NaCl, pH = 7.3).

Small unilamellar liposomes were obtained by sonication of the multilamellar liposomes dispersion (Branson Sonifier B12). The temperature was kept above T_c during sonication [5]. A circular (diameter 2 cm) vibrating inox electrode was employed to measure the surface potential [17–20]. An Ag electrode was used as reference. Fluorescence spectra were recorded with a differential spectrofluorimeter Focci MK 1.

TABLE I

STRUCTURE OF LOCAL ANESTHETICS

1, procaine; 2, tetracaine; 3, butacaine



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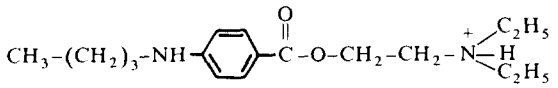

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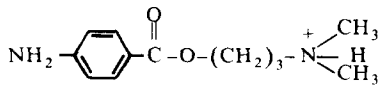

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Results and Discussion

1. Surface potential measurements

When an anesthetic is injected under a lipid monolayer spread at the air-water interface, the following interfacial reaction occurs:

$$D^+ \text{ (subphase)} + L^- \text{ (monolayer)} \rightleftharpoons DL \text{ (monolayer)} \quad (1)$$

where D^+ is the anesthetic, L^- the lipid anionic site and DL the complex.

From the association degree β:

$$\beta = [DL]/([DL] + [L^-]) \quad (2)$$

the equilibrium constant of Reaction 1 can be evaluated:

$$K_c = \frac{\beta}{1-\beta} \cdot \frac{1}{[D^+]_s} \quad (3)$$

where $[D^+]_s$ is the anesthetic concentration of D^+ at the interface.

$[D^+]_s$ is related to the bulk concentration $[D^+]_\infty$ through a Boltzmann distribution:

$$[D^+]_s = [D^+]_\infty \exp(-e\psi/kT) \quad (4)$$

e is the electronic charge, k the Boltzmann constant and ψ the electrostatic potential after complexation.

The electrostatic potential ψ (mV) associated to the lipid monolayer is given by the Gouy-Chapman theory. At 25°C [18,21]:

$$\psi = 50.4 \sinh^{-1}\left(134\sigma/\sqrt{c}\right) \quad (5)$$

where σ is the surface charge density in charge/Å^2 and c is the molar salt (univalent) concentration in the subphase. σ is directly related to the association degree. Indeed,

$$\sigma = (1-\beta)/A \quad (6)$$

A is the area occupied per lipid molecule (or per charged group) in the close packed state.

The experimental value of $[D^+]_s$, the anesthetic

concentration at the interface and β, the association degree allow the calculation of K_c. These values can be obtained by surface potential measurements. Indeed, the surface potential associated to a lipid monolayer spread on a subphase containing a drug can be described [20] by:

$$\Delta V = \frac{12\pi}{A}\mu_1(1-\beta) + \frac{12\pi}{A}\mu_2\beta + \psi \qquad (7)$$

where μ_1 and μ_2 are, respectively, the vertical component of the dipole moment (in mD) associated to the free and complexed lipid. A is the area occupied per lipid molecule in the monolayer, and ψ, the electrostatic potential. This relation is not limited to drug-lipid interaction but can be extended to other complexation processes (ionophore-ion [20]).

If no complex is formed at the interface ($\beta = 0$; $\psi = \psi_0$), Eqn. 7 becomes:

$$\Delta V_0 = \frac{12\pi}{A}\mu_1 + \psi_0 \qquad (8)$$

If only the lipid-anesthetic complex exists at the interface ($\beta = 1$, $\sigma = 0$, $\psi = 0$):

$$\Delta V_m = \frac{12\pi}{A}\mu_2 \qquad (9)$$

Combination of Eqns. 7, 8 and 9 gives:

$$\Delta V = \beta(\Delta V_m - \Delta V_0 + \psi_0) + \Delta V_0 - \psi_0 + \psi \qquad (10)$$

Several parameters of Eqn. 10 can be determined experimentally. Indeed, ΔV_0 is the ΔV value obtained with the uncomplexed lipid and ΔV_m, the ΔV value corresponding to a maximal lipid-anesthetic interaction. The determination of the ΔV evolution as a function of the $[D^+]_\infty$ bulk concentration allows to reach a concentration level where ΔV remains constant. This point is illustrated in Fig. 1 for the cardiolipin-butacaine systems where the surface potential reaches a constant value for butacaine concentrations superior to $6 \cdot 10^{-3}$ M. This means that cardiolipin-butacaine interaction is maximal but also that, after complexation, additional anesthetic molecules will not modify the surface potential. At a given temperature and ionic strength of the subphase, the following procedure of calculation will be used:

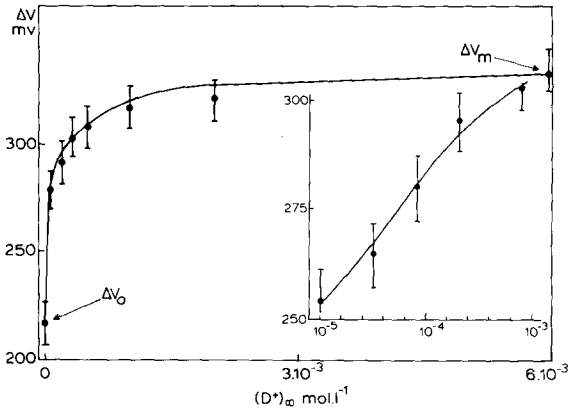

Fig. 1. Evolution of ΔV as a function of $[D^+]_\infty$, for the cardiolipin-butacaine system. $[NaCl] = 10^{-1}$ M, $\Delta V_0 = 220$ mV, $\Delta V_m = 335$ mV, pH = 6.6.

(1) For several values imposed to β (between 0 and 1), ψ is calculated from Eqns. 5 and 6.

(2) From the known values of ΔV_0 and ΔV_m (Fig. 1), from the ψ_0 calculated value (Eqns. 5 and 6 with $\beta = 0$) and from the ψ (see (1)) calculated value, ΔV is obtained from Eqn. 10.

(3) From the experimental curve $\Delta V = f[D^+]_\infty$ given in Fig. 1, $[D^+]_\infty$ associated to each value of the calculated ΔV can be obtained.

(4) Eqn. 4 allows to calculate $[D^+]_s$ from $[D^+]_\infty$ and ψ.

(5) K_c is calculated from $[D^+]_s$ and β values (Eqn. 3) (Table II).

The general procedure can however be simplified if the $\mu_1 = \mu_2$ condition is satisfied. Indeed, Eqns. 7 and 8 give:

$$\Delta V_0 - \Delta V = \psi_0 - \psi \qquad (11)$$

and if ΔV experimental reaches ΔV_m:

$$\Delta V_0 - \Delta V_m = \psi_0 \qquad (12)$$

Experimentally, this means that the difference between experimental values of ΔV_0 and ΔV_m must not differ significantly from ψ_0 (calculated from Eqns. 5 and 6 with $\beta = 0$). In these conditions, ψ is given by Eqn. 11 for each experimental ΔV value and β is obtained from Eqns. 5 and 6.

$[D^+]_s$ is evaluated from Eqn. 4, and from β and

TABLE II

CALCULATION OF K_c (BUTACAINE-CARDIOLIPIN SYSTEM) BY THE GENERAL PROCEDURE (SEE TEXT)

ΔV was not determined for $[D^+]_\infty < 10^{-5}$ M. $A = 58$ Å^2 (area per charged group in a close packed film); [NaCl] $= 10^{-1}$ M; $\psi_0 = -135$ mV; $\Delta V_0 = 220$ mV (Fig. 1); $\Delta V_m = 335$ mV (Fig. 1); pH = 6.6.

β (imposed)	$-\psi$ (mV) (Eqns. 5, 6)	ΔV (mV) (Eqn. 10)	$[D^+]_\infty$ (mol·l^{-1}) ($\times 10^5$)	$[D^+]_s$ (mol·l^{-1}) ($\times 10^4$) (Eqn. 4)	K_c (l·mol^{-1}) ($\times 10^{-3}$) (Eqn. 3)
0.60	90	253	1.0	3.6	4.2
0.70	77	264	2.5	5.4	4.3
0.80	60	279	8.0	8.8	4.5
0.85	47	291	20.0	13.1	4.3
0.90	35	302	45.0	18.2	4.9
0.95	18	318	200	41.0	4.6
1	0	335	–	–	–

$[D^+]_s$, K_c is obtained from Eqn. 3. This simplified approach has been used for the butacaine-cardiolipin system (Table III). It remains, however, that the $(\Delta V_0 - \Delta V_m)$ term is equal to -115 mV whereas ψ_0 calculated from Eqn. 5 gives -135 mV. This approximation supposes an overestimation of the ψ (Eqn. 11) and $[D^+]_s$ (Eqn. 4) values and explains the lower value of K_c obtained with the simplified procedure. If ΔV is determined with an accuracy of ± 5 mV, the mean K_c value ($1.4 \pm 0.4 \cdot 10^3$ l·mol^{-1}) was however in reasonable agreement with the mean K_c value obtained from the general procedure ($4.5 \pm 1 \cdot 10^3$ l·mol^{-1}). For these reasons, the simplified procedure was used to compare the interaction between several lipids (DL-α-dipalmitoylphosphatidylcholine, phosphatidylserine, cardiolipin and several anesthetics (procaine, butacaine, tetracaine).

Tables IV and V give the ψ, β, $[D^+]_s$ and K_c values obtained using this procedure. Butacaine and procaine (10^{-4} M) were injected under monolayers of cardiolipin, phosphatidylserine and DL-α-dipalmitoylphosphatidylcholine. Figs. 2 and 3 show the evolution of the experimental surface potential as a function of time for the systems used. Usually, a stable ΔV value is reached, 30 min after the

TABLE III

CALCULATION OF K_c (BUTACAINE-CARDIOLIPIN SYSTEM) BY THE SIMPLIFIED PROCEDURE (SEE TEXT)

The studied ΔV values were chosen in function of the results obtained in Table II. $A = 58$ Å^2 (area per charged group in a close packed film); [NaCl] $= 10^{-1}$ M; $\psi_0 = -135$ mV; pH = 6.6.

ΔV (mV) (exp.)	$[D^+]_\infty$ (mol·l^{-1}) ($\times 10^5$)	$-\psi$ (mV) (Eqn. 11)	β (Eqns. 5, 6)	$[D^+]_s$ (mol·l^{-1}) ($\times 10^4$) (Eqn. 4)	K_c (l·mol^{-1}) ($\times 10^{-3}$) (Eqn. 3)
253	1.0	102	0.50	5.9	1.7
264	2.5	91	0.60	9.5	1.6
279	8.0	76	0.70	16.7	1.4
291	20.0	64	0.77	25.8	1.3
302	45.0	53	0.83	37.4	1.3
318	200	37	0.89	87.8	0.9
335	–	20	–	–	–

TABLE IV

CALCULATION OF K_c FOR THE LIPID-BUTACAINE SYSTEM

$\Delta(\Delta V)=\Delta V_0-\Delta V$ is recorded after 30 min. $[NaCl]=10^{-2}$ M; $[D^+]_\infty=10^{-4}$ M. PS, phosphatidylserine. pH=6.6. A, area per molecule or per charged group in a close packed film.

Monolayer	A (Å^2)	$\Delta V_0-\Delta V$ (mV) (exp.)	ψ_0 (mV) (Eqns. 5, 6) ($\beta=0$)	ψ (mV) (Eqn. 11)	β (Eqns. 5, 6)	$[D^+]_s$ (mol·l^{-1}) (Eqn. 4)	K_c (l·mol^{-1}) (Eqn. 3)
DPPC	50	0	0	0	0	$1.0\cdot10^{-4}$	0
PS	65	−71	−187	−116	0.76	$1.0\cdot10^{-2}$	$3.2\cdot10^{2}$
Cardiolipin	58	−98	−193	−95	0.86	$4.4\cdot10^{-3}$	$1.4\cdot10^{3}$

anesthetic injection. No ΔV change was obtained when butacaine and procaine were injected under a neutral monolayer (dipalmitoylphosphatidylcholine) meanwhile the ΔV increase (with charged lipids) demonstrated the existence of a lipid anionic site-anesthetic interaction.

Fig. 4 indicates that the ΔV experimental value of the neutral DPPC monolayer is modified in presence of tetracaine. The ΔV increase obtained in the case of the neutral monolayer is related to a positive ψ value associated to the interaction of tetracaine with the lipid:

$$D^+ \text{ (subphase)} + L^0 \text{ (monolayer)} \rightleftharpoons DL^+ \text{ (monolayer)} \qquad (13)$$

where L^0 is the neutral lipid.

The equilibrium constant of this reaction is given by Eqn. 3. The potential ψ is correlated to σ by a relation similar to Eqn. 5 but the positive charge density is in the present case defined by:

$$\sigma=\beta/A \qquad (14)$$

where β is the association degree between the neutral lipid and the positively charged drug.

ψ value is obtained from Eqn. 11 with $\psi_0=0$ (the monolayer is uncharged if no anesthetic is injected in the subphase). From ψ, one obtains the β value (Eqns. 5 and 14) and the $[D^+]_s$ value (Eqn. 4). Finally, K_c is calculated from Eqn. 3. Table VI summarizes the K_c values obtained for dipalmitoylphosphatidylcholine, phosphatidylserine and cardiolipin. The K_c values obtained for the DPPC-tetracaine system suppose however that the interaction is not only electrostatic.

2. Fluorescence measurements

Results obtained by the surface potential technique provided quantitative data about electrostatic interactions between negatively charged

TABLE V

CALCULATION OF K_c FOR THE LIPID-PROCAINE SYSTEM

The conditions are the same as those described in Table IV.

Monolayer	A (Å^2)	$\Delta V_0-\Delta V$ (mV) (exp.)	ψ_0 (mV) (Eqns. 5, 6) ($\beta=0$)	ψ (mV) (Eqn. 11)	β (Eqns. 5, 6)	$[D^+]_s$ (mol·l^{-1}) (Eqn. 4)	K_c (l·mol^{-1}) (Eqn. 3)
DPPC	50	0	0	0	0	$1.0\cdot10^{-4}$	0
PS	65	−25	−187	−162	0.40	$6.2\cdot10^{-2}$	10.8
Cardiolipin	58	−25	−193	−168	0.42	$7.0\cdot10^{-2}$	10.3

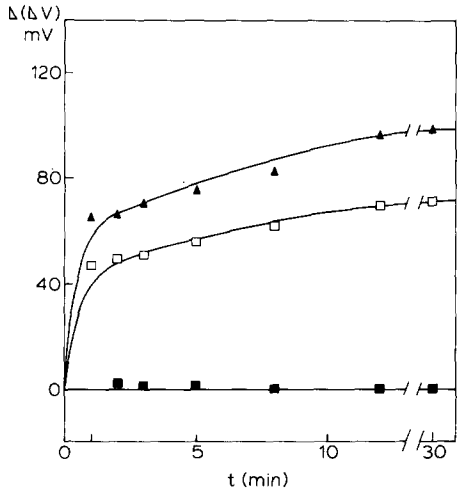

Fig. 2. Evolution of $\Delta(\Delta V)=\Delta V_0-\Delta V$, as a function of time, for the lipid-butacaine system. [NaCl]$=10^{-2}$ M, $[D^+]_\infty=10^{-4}$ M, pH=6.6. ▲, cardiolipin; □, phosphatidylserine; ■, DL-α-dipalmitoylphosphatidylcholine.

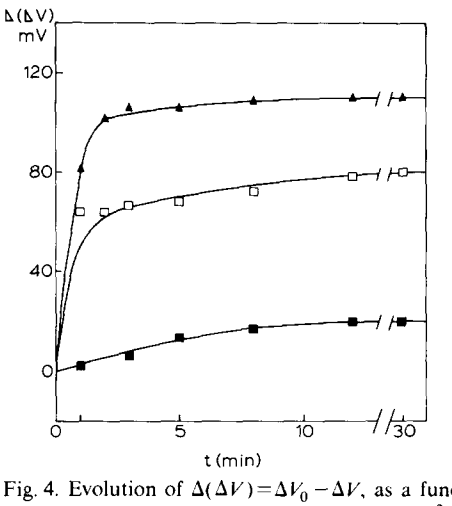

Fig. 4. Evolution of $\Delta(\Delta V)=\Delta V_0-\Delta V$, as a function of time, for the lipid-tetracaine system. [NaCl]$=10^{-2}$ M, $[D^+]_\infty=10^{-4}$ M, pH=6.6. ▲, cardiolipin; □, phosphatidylserine; ■, DL-α-dipalmitoylphosphatidylcholine.

phospholipids and positively charged anesthetics. We have shown that in the case of procaine and butacaine electrostatic interactions with phospholipids were essential for the binding. It remains necessary however to elucidate how the interaction between anesthetic molecules and lipid acyl chains can influence the binding. Fluorescence properties of anesthetics can be used for this purpose. Indeed, the maximum emission wavelength of the fluorescence is strongly dependent on the dielectric constant of the medium surrounding the chromophores. Therefore, the fluorescence can be used to define the dielectric constant of the medium in which the dye is embedded.

Shinitzky [22] has evaluated the dielectric constant profil of the bilayer by this method. Fig. 5 shows the position of the maximum emission wavelength (λ_{max}) of each anesthetic as a function of the dielectric constant (ϵ). Media of different

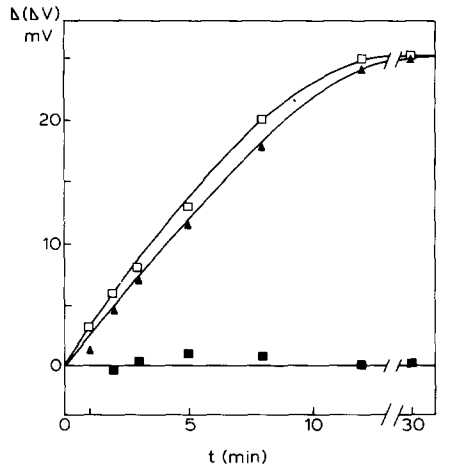

Fig. 3. Evolution of $\Delta(\Delta V)=\Delta V_0-\Delta V$, as a function of time, for the lipid-procaine system. [NaCl]$=10^{-2}$ M, $[D^+]_\infty=10^{-4}$ M, pH=6.6. ▲, cardiolipin; □, phosphatidylserine; ■, DL-α-dipalmitoylphosphatidylcholine.

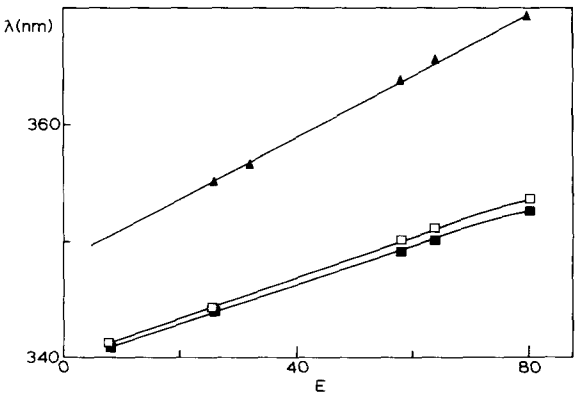

Fig. 5. Evolution of λ_{max} as a function of the dielectric constant ϵ of the medium, for tetracaine (▲), butacaine (□) and procaine (■).

TABLE VI

CALCULATION OF K_c FOR THE LIPID-TETRACAINE SYSTEM

The conditions are the same as those described in Table IV.

Monolayer	A ($Å^2$)	$\Delta V_0 - \Delta V$ (mV) (exp.)	ψ_0 (mV) (Eqns. 5, 6) ($\beta = 0$)	ψ (mV) (Eqn. 11)	β (Eqns. 5, 6) (or 5, 14)	$[D^+]_s$ ($mol \cdot l^{-1}$) (Eqn. 4)	K_c ($l \cdot mol^{-1}$) (Eqn. 3)
DPPC	50	−20	0	20	0.015	$4.5 \cdot 10^{-5}$	$3.3 \cdot 10^2$
PS	65	−80	−187	−107	0.80	$6.2 \cdot 10^{-3}$	$6.5 \cdot 10^2$
Cardiolipin	58	−110	−193	−83	0.89	$2.6 \cdot 10^{-3}$	$3.0 \cdot 10^3$

dielectric constant were prepared as follows: aqueous buffer ($\epsilon = 80$), buffer/methanol (2:1, v/v) ($\epsilon = 66$), buffer/methanol (1:1, v/v) ($\epsilon = 58$), methanol ($\epsilon = 33$) and ethanol ($\epsilon = 24$).

In a second step, the anesthetic fluorescence spectra were measured in presence of different phospholipids. In order to reduce scattered light, small unilamellar liposomes were used [7]. Evolution of λ_{max} for increasing liposome concentrations is reported in Figs. 6 to 8. A constant value of λ_{max} for increasing liposome concentrations supposes a maximal incorporation of the anesthetic in the lipid core. Reporting this value of λ_{max} in Fig. 5, the dielectric constant (ϵ) of the lipid environment surrounding the anesthetic can be obtained. ϵ values are reported in Table VII.

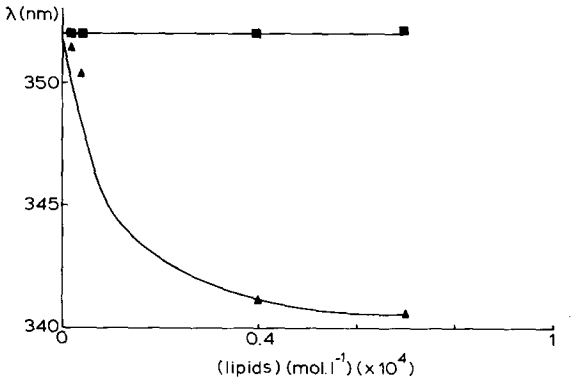

Fig. 7. Evolution of λ_{max} as a function of the lipidic concentration for the lipid-butacaine system. ■, DL-α-dipalmitoylphosphatidylcholine; ▲, cardiolipin. [NaCl] = 10^{-1} M, pH = 7.3.

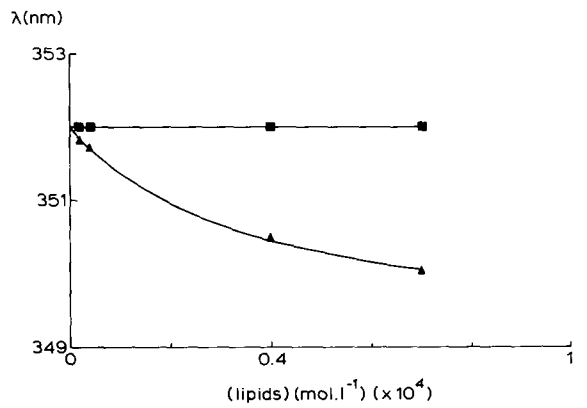

Fig. 6. Evolution of λ_{max} as a function of the lipidic concentration for the lipid-procaine system. ■, DL-α-dipalmitoylphosphatidylcholine; ▲, cardiolipin. [NaCl] = 10^{-1} M, pH = 7.3.

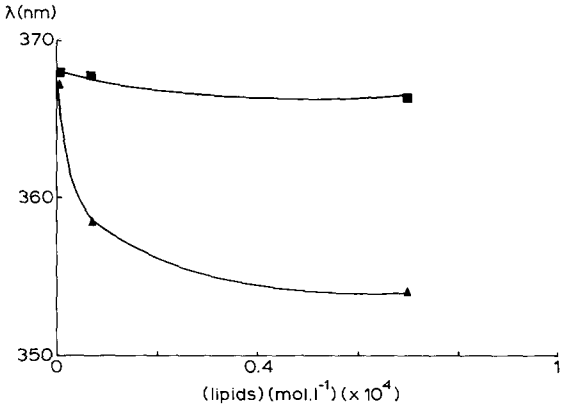

Fig. 8. Evolution of λ_{max} as a function of the lipidic concentration for the lipid-tetracaine system. ■, DL-α-dipalmitoylphosphatidylcholine; ▲, cardiolipin. [NaCl] = 10^{-1} M, pH = 7.3.

TABLE VII

COMPARISON OF ϵ VALUES AND OF K_c VALUES FOR DIFFERENT LOCAL ANESTHETICS-LIPIDS SYSTEM

Anesthetic	Lipid			
	DPPC		Cardiolipin	
	K_c ($l \cdot mol^{-1}$)	ϵ	K_c ($l \cdot mol^{-1}$)	ϵ
Butacaine	0	80	$1.4 \cdot 10^3$	8
Procaine	0	80	10.3	66
Tetracaine	$3.3 \cdot 10^2$	66	$3.0 \cdot 10^3$	12

Table VII shows the correlation between the lipid-anesthetic association constant and the anesthetic capacity to interact with lipids: a high anesthetic-lipid association constant is indeed associated to a strong interaction of the anesthetic molecule with the lipid core (tetracaine-cardiolipin, butacaine-cardiolipin). A quantitative comparison of K_c and ϵ would suppose the knowledge of the area occupied per lipid in the sonicated liposomes. These data are not available for all the systems used in the present study.

Conclusions

We describe a method for the evaluation of the anionic lipid-drug association constant based on surface potential measurements. The procedure could be extended to any kind of lipid-drugs interaction. Other model membranes have been used to determine such association constants [1,2]. However, their use remains limited by the unstability of the system (planar lipid bilayers) or by the necessity to use lipids having a transition temperature in a usual temperature range [2]. Local anesthetic-lipid interactions depend on the charge of each component but also on the physical state of the lipid. However, it must be kept in mind that high drug-lipid association constants can be obtained without drug penetration into the lipid core. Adriamycin for example has a very high affinity for cardiolipin [5] but does not penetrate the lipid bilayer.

It has been proposed that two molecules of adriamycin in interaction with one molecule of cardiolipin are sufficiently close to form a card-pack dimer [5]. This structure would stabilize the complex. A clear understanding of the role of the drug position in the lipid layer on the interaction requires a systematic study of other systems but also the possibility to determine the position of the drug in the lipid layer. We recently developed a procedure of theoretical conformational analysis permitting the location of the mean position of the lipid and of the drug in the lipid matrix (Brasseur R. and Ruysschaert, J.M., unpublished data). Another work is in progress to determine the position of the three anesthetics in the lipid monolayer.

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