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Binding of lipophilic cations to the liposomal membrane: thermodynamic analysis

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Lipophilic ions are widely used as probes for measuring membrane potentials. Since binding of the probes to the membrane interferes with the accurate estimation of the membrane potential, it is necessary to clarify the characteristics of probe binding to membranes. The present paper deals with the binding of lipophilic cations to liposomes. The results can be summarized as follows: (1) The binding of triphenylmethylphosphonium, its homologues and tetraphenylphosphonium to liposomes of dipalmitoylphosphatidylcholine followed the Langmuir adsorption isotherm. (2) Spin-labeled lipophilic cations were synthesized and the binding to liposomes of egg phosphatidylcholine was examined. The binding also followed the Langmuir adsorption isotherm. The dissociation constant (the concentration giving half-maximal binding), *K*, was independent of the temperature, indicating that the binding is entropy-driven. (3) The binding was influenced by the fluidity of the membrane. Except in the case of triphenylmethylphosphonium ($TPMP^+$), *K* and *A* (maximum amounts of binding) increased above the transition temperature. In other words, above the phase transition temperature the binding affinity is decreased, while maximum amounts of binding are increased for all phosphoniums used except $TPMP^+$.

Introduction

It is well known that lipophilic ions are used as probes to estimate the membrane potentials of bacteria and organelles which are too small to allow the use of micro-electrodes [1–6]. The principle of the estimation of membrane potentials using lipophilic ions is that these ions can permeate cell membranes freely and at equilibrium, distributing between the inside and outside of cells according to the Nernst equation. Therefore, estimation of the probe concentration inside versus outside the cell enables us to calculate the membrane potential. The principle seems simple and reliable. However, an ambiguity has been pointed out, which has arisen from the binding of the probe to the cell membrane and/or intracellular constituents [7–16], and this binding usually results in overestimation of the membrane potential.

The present paper deals with the binding of various lipophilic cations to the liposomal membrane. The lipophilic cations used are tetraphenylphosphonium (TPP^+), triphenylmethylphosphoni-

Lipophilic cations used: triphenylmethylphosphonium ($TPMP^+$), triphenylethylphosphonium ($TPEP^+$), triphenylpropylphosphonium ($TPPP^+$), triphenylbutylphosphonium ($TPBP^+$), triphenylamylphosphonium ($TPAP^+$) and tetraphenylphosphonium (TPP^+).

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um ($TPMP^+$), and its homologues $(Phe)_3\text{-}P^+\text{-}(CH_2)_n\text{-}CH_3$ ($n = 0$–5) (the names and their abbreviations are given in a footnote). In addition to these probes, spin-labeled lipophilic cations were used. Since the EPR-signal from the bound population is distinctly different from that of the free population, it is possible to observe both bound and free populations simultaneously in the presence of membranes.

Materials and Methods

Preparation of liposomes

Lipids used were dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylcholine. DPPC was purchased from Sigma and used without further purification. Egg phosphatidylcholine was purified with silica-gel chromatography (chloroform/methanol, 10 : 1). A single spot was observed on a silica-gel thin-layer chromatogram (chloroform/methanol/28% ammonia/water, 120 : 80 : 10 : 5).

Liposomes were prepared by sonication (20 kHz, 120 W for 15–30 min) in 100 mM NaCl, 10 mM Hepes at pH 7.0 under a stream of nitrogen, followed by ultra-centrifugation (100 000 × g, 60 min) to remove large liposomes. Liposomal phosphate was assayed using the method of Bartlett [17].

Synthesis of spin-labeled lipophilic cations

The spin-probes used are shown in Fig. 1. Probe 1 was synthesized in accordance with the method of Cafiso and Hubbell [18] with some modifications. Probe II was synthesized by Molecular Probes and used without further purification. Probe III was synthesized by the method of Mehlhorn and Packer [19].

Measurement of probe-binding with a selective electrode

Binding of various lipophilic ions to liposomes was measured with the use of electrodes selective for lipophilic ions. Construction of electrodes and the apparatus used were as described previously [20]. Since the electrode responds only to the free ions, the change in the electrode potential observed on addition of liposomes is caused by depletion of the free probe due to binding to the membranes. The change in the electrode potential due to the dilution resulting from addition of the liposome suspension was corrected. The volume ratio of the added liposome suspension to the total volume was less than 5%. The electrode potential at equilibrium also enabled us to calculate the equilibrium concentration of the free lipophilic cations.

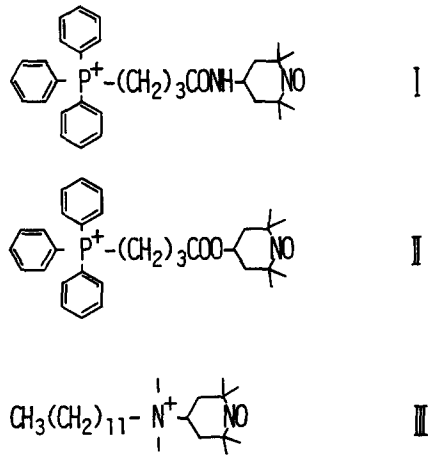

Fig. 1. Spin-probes used.

Measurement of probe-binding with EPR

Binding of spin-labeled lipophilic ions was measured essentially with the method of Cafiso and Hubbell [18,21]. The EPR signal was obtained with JES-FE3X (Nihon Denshi, Tokyo) with 5 mW of microwave (9.20 GHz) and 100 kHz, 1 G of modulation. The magnetic field was 3290 ± 50 G. Temperature was controlled with a thermostated N_2 stream.

Results and Discussion

Binding follows the Langmuir equation

Fig. 2 shows the binding curves of various lipophilic ions to DPPC liposomes at 30 and 45 °C in 100 mM NaCl. The order of the amount of binding is $TPMP^+ < TPEP^+ < TPBP^+ < TPP^+ < TPAP^+$ at both temperatures, and this order corresponds to that of the length of the hydrocarbon chains of the $TPMP^+$ homologues. The same order was observed in the binding to the membrane of

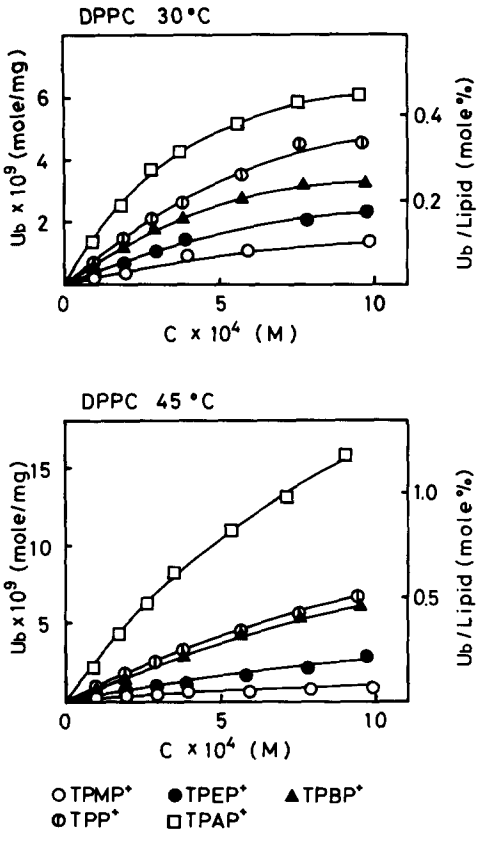

Fig. 2. The amount of binding, U_b, is plotted against the free concentration of various phosphonium cations. The medium was 100 mM NaCl, 10 mM Hepes-NaOH (pH 7.0). Temperature of the upper graph is 30 °C and that of the lower is 45 °C. ○, $TPMP^+$; ●, $TPEP^+$; ▲, $TPBP^+$; ⊕, TPP^+; □, $TPAP^+$.

Halobacterium halobium [22,23]. As the concentration of the ion increased, binding increased and leveled off, suggesting that the binding follows the saturation isotherm given by the Langmuir equation. Therefore, we assume that

$$U_b = AC/(K + C) \quad (1)$$

where A, K and C represent the maximum amount of binding, the dissociation constant and the free concentration of lipophilic ions, respectively. Rearrangement of Eqn. 1 yields

$$C/U_b = K/A + C/A \quad (2)$$

showing that the values of K and A are determined from the plot of C/U_b against C. One example of such a plot is shown in Fig. 3. We obtained straight lines for all probes except $TPMP^+$ and $TPEP^+$, whose amounts of binding were small. The values of A and K were determined with the aid of non-linear square regression and are listed in Table I. With the increase of the length of the hydrocarbon chain of $TPMP^+$ homologues, the value of K decreases, indicating a tighter binding and a larger A value. A similar tendency was observed for the membrane of *Halobacterium halobium* [22] and mitochondria (unpublished data).

We should pay attention to the change in surface potential caused by the binding of lipophilic cations. The change in the Gibbs free energy due to the binding is composed of two terms: a non-electrical part and an electrical part [24]. The surface potential contributes significantly to the latter. A change in the surface potential leads to a change in the dissociation constant of the lipophilic cation binding. The surface potential is diminished in high salt solutions. On the assumption that one lipid occupies 70 Å^2 (10^{-16} cm^2) in the liposome surface [26] and that the ratio of molecules of the bound cation to those of lipids is 1% (see Fig. 2), the surface potential change due to the binding is calculated to be about 3 mV in 100 mM NaCl. The change in free energy due to the change in surface potential caused by the

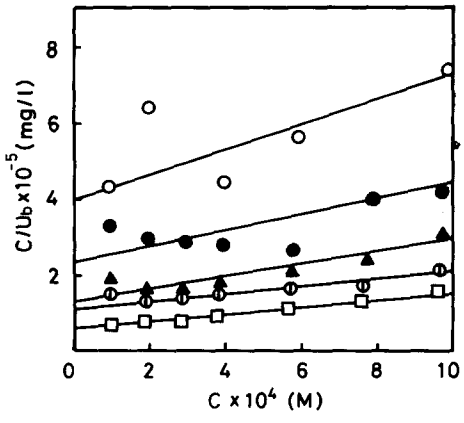

Fig. 3. Determination of A and K according to Eqn. 2. The data are taken from the upper graph of Fig. 2 (30 °C). Notations are the same as in Fig. 2.

TABLE I

BINDING PARAMETERS OF VARIOUS PROBES TO DPPC LIPOSOME

The medium was 100 mM NaCl, 10 mM Hepes-NaOH (pH 7.0).

Probe	30 °C		45 °C	
	K (mol/l)	A (mol/mg)	K (mol/l)	A (mol/mg)
$TPMP^+$	$1.05 \cdot 10^{-3}$	$2.84 \cdot 10^{-9}$	$6.03 \cdot 10^{-4}$	$1.31 \cdot 10^{-9}$
$TPEP^+$	$9.46 \cdot 10^{-4}$	$4.84 \cdot 10^{-9}$	$2.16 \cdot 10^{-3}$	$7.86 \cdot 10^{-9}$
$TPBP^+$	$6.56 \cdot 10^{-4}$	$5.60 \cdot 10^{-9}$	$1.80 \cdot 10^{-3}$	$1.80 \cdot 10^{-8}$
$TPAP^+$	$4.62 \cdot 10^{-4}$	$9.25 \cdot 10^{-9}$	$1.39 \cdot 10^{-3}$	$3.97 \cdot 10^{-8}$
TPP^+	$1.08 \cdot 10^{-3}$	$1.00 \cdot 10^{-8}$	$2.19 \cdot 10^{-3}$	$2.23 \cdot 10^{-8}$

binding is 0.065 kcal/mol, while the free energy change calculated from K is several kcal/mol. This implies that the change in the surface potential may be neglected in the present system. It is noted that this does not imply that the value of K does not contain the contribution of the electrical part.

Enthalpy and entropy changes of binding

Fig. 2 indicates that amounts of binding at 45 °C are much larger than those at 35 °C for all lipophilic ions used, especially for the lipophilic ions having longer hydrocarbon chains. Since DPPC shows a phase transition at about 41 °C, the temperature-dependence of the binding could partially be attributed to the phase transition (this will be discussed below). In order to bypass the problem of phase transition, egg phosphatidylcholine was used which remains fluid in the temperature range examined.

Binding of the spin-probes to liposomes of egg phosphatidylcholine followed the Langmuir equation and is similar to the binding to DPPC for a given temperature. Analysis with Eqn. 2 gave a fairly good straight line (determination coefficient was 0.991 or more) for all data at varying temperatures. Binding parameters at varying temperatures are listed in Table II. The K and A values of probe I (amide type) are about 10-times larger than those of probe II (ester type); probe I has low binding affinity but has large maximum amounts of binding. Probe III shows a very small value of K compared with the other two spin-probes, and the existence of two binding sites is shown.

TABLE II

BINDING PARAMETERS OF SPIN-PROBE I, II AND III TO LIPOSOMES OF EGG PHOSPHATIDYLCHOLINE

The medium was 100 mM NaCl, 10 mM Hepes-NaOH (pH 7.0). The values of A and K were determined with Eqn. 1.

T (°C)	K (mol/l)		A (mol/mg)	
Probe I				
5	$1.18 \cdot 10^{-3}$		$8.43 \cdot 10^{-8}$	
15	$1.29 \cdot 10^{-3}$		$1.64 \cdot 10^{-7}$	
25	$1.12 \cdot 10^{-3}$		$1.70 \cdot 10^{-7}$	
35	$1.28 \cdot 10^{-3}$		$1.45 \cdot 10^{-7}$	
Probe II				
5	$1.34 \cdot 10^{-4}$		$2.08 \cdot 10^{-8}$	
15	$1.58 \cdot 10^{-4}$		$2.39 \cdot 10^{-8}$	
25	$1.79 \cdot 10^{-4}$		$2.69 \cdot 10^{-8}$	
35	$1.81 \cdot 10^{-4}$		$2.76 \cdot 10^{-8}$	
Probe III	(high) [a]	(low) [a]	(high) [a]	(low) [a]
5	$6.62 \cdot 10^{-6}$	$7.64 \cdot 10^{-5}$	$1.72 \cdot 10^{-7}$	$4.72 \cdot 10^{-7}$
15	$6.31 \cdot 10^{-6}$	$1.06 \cdot 10^{-4}$	$1.71 \cdot 10^{-7}$	$5.43 \cdot 10^{-7}$
25	$5.75 \cdot 10^{-6}$	$1.24 \cdot 10^{-4}$	$1.67 \cdot 10^{-7}$	$5.83 \cdot 10^{-7}$
35	$5.76 \cdot 10^{-6}$	$1.26 \cdot 10^{-4}$	$1.66 \cdot 10^{-7}$	$5.88 \cdot 10^{-7}$

[a] High and low indicate the high-affinity and low-affinity site, respectively.

The parameter of K in Eqn. 2 is related to the change in the Gibbs free energy associated with the binding as follows:

$$\Delta G = -RT \ln(1/K)$$

From the usual thermodynamic treatment, we can estimate the changes in the enthalpy and entropy. These are listed in Table III, indicating that entropy contributes significantly to the change in Gibbs free energy while the enthalpy term is almost zero, although small enthalpy changes of the low-affinity binding site of probe III were found. This is characteristic of the hydrophobic effect [26]. Thermodynamic analysis of the binding of lipophilic cation to liposomes was studied also by Flewelling and Hubbell [27] who assumed that binding is proportional to the concentration of the free lipophilic cation. They found small positive enthalpy changes (3.5–3.9 kcal/mol) and large positive entropy changes (21–27 cal/mol per deg). Our data are somewhat different from theirs in

TABLE III

THERMODYNAMICAL PARAMETERS IN THE BINDING OF LIPOPHILIC CATIONS TO LIPOSOMES

IIIa and IIIb stand for the high- and low-affinity binding site of probe III, respectively.

Probe	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol per deg)
I	−4.0	0	13.5
II	−5.1	0	17.1
IIIa	−7.1	0	23.9
IIIb	−5.3	−2.3	10.1

that we found that enthalpy changes are almost zero. In spite of this difference, both studies lead to the conclusion that binding is entropy-driven.

Effect of phase transition of bilayer membrane on the binding of lipophilic ions

In order to investigate the effect of the phase transition of bilayer membranes on the binding of lipophilic ions, DPPC liposomes were employed. The amounts of binding of the spin-probes to the membrane were measured at varying temperatures when the total (free and bound) concentrations of probes were fixed at 100 μM (data not shown). We observed an appreciable increase in the amounts of binding above the phase transition temperature; with probe II, the amount of binding in the fluid state was 5.5-times as much as that in the solid state. Fig. 2 also shows that amounts of binding in the fluid state are larger than in the solid state for various phosphonium ions.

Some compounds are known whose binding to the membrane is reflected by the fluidity of the membrane: one of them is TEMPO, which is widely used as an indicator for the fluidity of the membrane [28], since it is distributed more into the membrane phase when the membrane is more fluid. The thermodynamic analysis, however, was not carried out as far as we are aware.

The thermodynamic analysis of the binding of lipophilic cations (see Table I) reveals that except in the case of $TPMP^+$ the values of K increased above the phase transition temperature, meaning a decrease in binding affinity. The maximum amounts of binding (A) increased above this temperature, except for $TPMP^+$, which showed an opposite variation of A. Thus, $TPMP^+$ behaves differently from other lipophilic cations. The reason is not clear at present and needs further investigation. To solve the problem, a detailed thermodynamic analysis of binding of these lipophilic ions to the liposome above and below the phase transition is needed.

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