STRUCTURAL REQUIREMENT FOR THE RAPID MOVEMENT OF CHARGED MOLECULES ACROSS MEMBRANES

Experiments with Tetraphenylborate Analogues

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ABSTRACT Charge-pulse experiments were performed in the presence of structural analogues of tetraphenylborate (TPB) on membranes made of dioleoyl phosphatidylethanolamine and dioleoyl phosphatidylcholine. The analysis of the experimental results using a previously proposed model allowed the calculation of the partition coefficient, β, and of the translocation rate constant, k_i. The temperature dependence of the partition coefficients was used to calculate the thermodynamics of the adsorption of the lipophilic ions to the membranes. The analysis of the translocation rate constants obtained at different temperatures yielded detailed information on the free energy of the TPB-analogues within artificial lipid bilayer membranes, and on the activation energy of the translocation rate constants. The adsorption of the different TPB-analogues to the membranes was only slightly affected by their structure, whereas a dramatic influence of the structure on the free energy of the lipophilic ions within the membranes was observed. The free energy of the ions in the membranes decreased from triphenylcyanoborate (TPCB) to tetrakis(3-trifluoromethylphenyl)borate (TTFPB) by more than 31 kJ/mol (7.4 kcal/mol). This could be concluded from the observed increase in the translocation rate constant by almost six orders of magnitude. The change of the free energy in the membrane was used for the estimation of an effective radius of the TPB-analogues with respect to TPB.

INTRODUCTION

In recent years the influence of the structure of biological and artificial membranes on the transmembrane charge transport was investigated using charged probe molecules such as several lipophilic ions and carrier molecules (Szabo, 1975; Andersen et al., 1978; Benz and Cros, 1978; Benz and Gisin, 1978; Benz and Conti, 1981; Benz and Nonner, 1981, Dilger and Benz, 1985; Flewelling and Hubell, 1986*a* and *b*). The studies with lipophilic ions yielded detailed information about the electrical properties of membranes such as surface potential, dipole potential, and Born charging energy (Andersen and Fuchs, 1975; Benz and Läuger, 1977; Pickar and Benz, 1978; Flewelling and Hubell, 1986*a* and *b*). The basic difference between lipophilic ions and small charged molecules is that the former have a high partition coefficient in favor of the membranes. Furthermore, lipophilic ions readily permeate artificial and biological membranes. Both properties are caused by the large hydrocarbon moiety of the lipophilic ions, which favors the partitioning and leads to a strong delocalization of the charge, thus reducing the Born charging energy of the ions within the membrane. The kinetics of the transport of lipophilic ions across membranes is well understood on the basis of a simple model which assumes an adsorption-desorption reaction as the first step. The transport within the membranes was successfully explained by a two-state model of the second step (Ketterer, Neumke and Läuger, 1971; Andersen and Fuchs, 1975; Benz et al., 1976).

Prominent examples of lipophilic ions are dipicrylamine (DPA) and tetraphenylborate (TPB)[1] (Ketterer et al., 1971; Bruner, 1975; Andersen and Fuchs, 1975; Szabo, 1975; Benz et al., 1976). Both lipophilic ions have been used in a large number of studies. Comparing the transport properties of these two lipophilic ions it was found that despite the symmetry and the considerable radius of TPB, DPA has a translocation rate constant, k_i, that is about 40 times larger than that of TPB. Furthermore, DPA showed a somewhat larger partition coefficient for adsorption to biological and artificial membranes (Benz et al., 1976; Benz and Conti, 1981; Benz et al., 1984). Neither effect is easy to understand from a comparison of the structure of these lipophilic ions.

In this publication the influence of the structure of the

[1] *Abbreviations used in this paper:* DPA, dipicrylamine; TPCB, triphenylcyanoborate; TPB, tetraphenylborate; TPP, tetraphenylphosphonium; TEB, tetraethylborate; TFPB, tetrakis(4-fluorophenyl)borate; TCPB, tetrakis(4-chlorophenyl)borate; TTFPB, tetrakis(3-trifluoromethylphenyl)borate.

lipophilic ions on their adsorption to lipid bilayer membranes and on their translocation rate constant was investigated in detail. For this purpose lipid bilayer experiments were performed with four different TPB analogues. In the first analogue one of the phenyl rings is replaced by a cyano-group (Fig. 1). In the other three analogues the phenyl rings are replaced by ethyl-, 4-fluorophenyl-, 4-chlorophenyl-, or 3-trifluoromethylphenyl groups (see Fig. 1). The results presented here indicate that the additional groups attached to the phenyl rings have a substantial influence on the translocation rate constants of the tetraphenylborate analogues. This influence may be explained on the basis of an increase of the effective radii of the lipophilic ions. In another set of experimental conditions the influence of temperature on the transport parameters of the tetraphenylborate analogues was studied. Here the influence of the structural changes was relatively small. The charge-pulse technique was used for all experiments because of its superior time resolution and because it causes the minimum electrical perturbation of the lipid bilayer membranes (Benz et al., 1976).

MATERIALS AND METHODS

Black lipid bilayer membranes were formed from a 1% solution of dioleoyl phosphatidylcholine or dioleoyl phosphatidylethanolamine (Avanti Biochemicals, Birmingham AL) in *n*-decane (Fluka AG, Buchs, Switzerland). The membranes were formed across circular holes with 1–2 mm diameter in the wall separating two aqueous compartments in a Teflon cell. The 1 M NaCl (E. Merck, Darmstadt, FRG) solutions were prepared using twice-distilled water. Experiments were performed at 5, 25, and 45°C. The tetraphenylborate analogues were added to the aqueous phase in the form of concentrated (10^{-3} to 10^{-4} M) solutions in ethanol to give final concentrations in the aqueous solutions between 10^{-8} and 10^{-7} M. These concentrations were chosen to obtain a linear relationship between the concentrations of the lipophilic ions in the aqueous phase and in the membrane (Benz et al., 1976; Wulf et al., 1976), and to avoid the formation of "boundary potentials" by the adsorption of the lipophilic ions (McLaughlin, 1977).

To reach partition equilibrium for the adsorption of the tetraphenylborate analogues to the bilayers, we carried out all kinetic experiments at least 20 min after the membranes had completely turned black with the exception of the experiments in the presence of tetraethylborate (TEB). This compound was not as stable as the others (which were stable for at least 5 h) in the aqueous solution and showed rapid hydrolyzation. It was added in concentrated ethanolic solution directly to the aqueous phase bathing black lipid bilayer membranes while stirring. The experiments were performed after equilibration of the lipophilic ion in the aqueous phase (1–2 min). The following lipophilic ions were used in this study. Triphenylcyanoborate (TPCB) and tetrakis(3-trifluoromethylphenyl)borate (TTFPB) were a generous gift of Dr. Ross W. Flewelling. Tetrakis(4-fluorophenyl)borate (TFPB) and tetrakis(4-chlorophenyl)borate (TCPB) were generously supplied by Dr. Shigeru Itoh, National Institute, Japan. Tetraphenylborate (TPB) and tetraethylborate (TEB) were obtained from E. Merck and Ferak, Berlin, FRG, respectively. The lipophilic ions were pure and were used as the sodium salts.

The charge pulse experiments were carried out as described previously (Benz and Zimmermann, 1983). In brief, one Ag/AgCl electrode was connected to a fast commercial pulse generator (Philips PM 5712) through a fast diode (reverse resistance > 10^{11} Ω) and the other electrode grounded. A resistor of 0.1, 1, or 10 MΩ was introduced between the two electrodes to define a passive RC-time constant for the membrane. The voltage between these two electrodes (maximum voltage, 10 mV) was measured with a high-input-resistance, fast voltage amplifier based on a Burr Brown operational amplifier and a digital storage oscilloscope (Nicolet 4094). The time resolution of the detection system was of the order of 100 ns. The voltage relaxations were analyzed with a HP 98580AD computer and HP 9862 plotter. They could always be fitted to two exponential relaxations (Benz and Zimmermann, 1983).

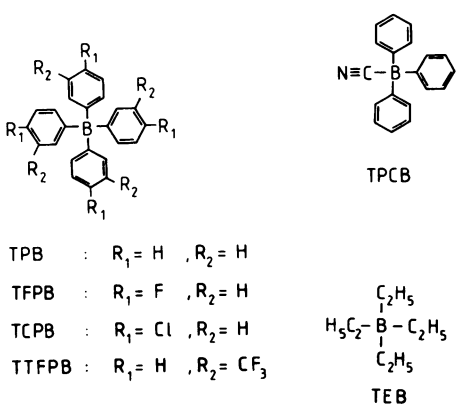

FIGURE 1 Structure of the tetraphenylborate analogues used in this study.

Theory

The theory of the movement of lipophilic ions across lipid bilayers and biological membranes was given in full detail in previous publications (Ketterer et al., 1971; Benz et al., 1976; Benz and Conti, 1981). Here we will only summarize the basic assumptions and list the equations which allow the calculation of the transport parameters from the experimental results. It is assumed that the lipophilic ions are adsorbed to deep free energy minima on both sides of the membrane with a total concentration of N_t per unit surface (partition coefficient $\beta = N_t/2c$, with c being concentration of the lipophilic ions in the aqueous phase). For symmetry reasons (identical solutions on the two sides of the membranes and a symmetrical bilayer) it is assumed that at zero membrane potential the lipophilic ions are equally distributed between the two membrane–solution interfaces and cross the intermediate free energy barrier with the same rate constant, k_i, in either direction. Under these conditions the shape of the barrier has no influence on the characteristics of the voltage relaxations measured in a charge pulse experiment as long as the initial membrane potential is much smaller than 25 mV (Benz and Zimmermann, 1983). Finally we neglect the exchange of lipophilic ions between the membrane and the aqueous phase during a single relaxation experiment because it is rate limited by slow aqueous diffusion (Benz et al., 1976) and has characteristic times much longer than the passive RC-time constant, τ_m, of the lipid bilayer membranes.

In a charge pulse experiment the system is in equilibrium at times $t < 0$ and the membrane capacitance is charged instantaneously at $t = 0$ to an initial voltage V_o (≪25 mV). The decay of the membrane voltage with time, $V(t)$, is given by the sum of two exponential relaxations.

$$V(t) = V_o\,[a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)], \qquad (1)$$

where a_1, a_2 ($=1 - a_1$), τ_1, and $\tau_2 > \tau_1$ are known functions of k_i, N_t, and τ_m (Benz and Conti, 1981; Benz and Zimmermann, 1983). The first (fast) relaxation process reflects the displacement of the lipophilic ions as a consequence of the suddenly applied voltage, whereas the second (slow) voltage relaxation represents the slow discharge of the membrane through the external resistor and the redistribution of charges within the membrane (Benz and Conti, 1981). τ_2 is always longer than the passive RC-time constant of the membrane (i.e., $\tau_2 > \tau_m$) because the lipophilic ions contribute to the effective capacity of the membrane (Benz, R., unpublished results). The inverse relations between the relaxation param-

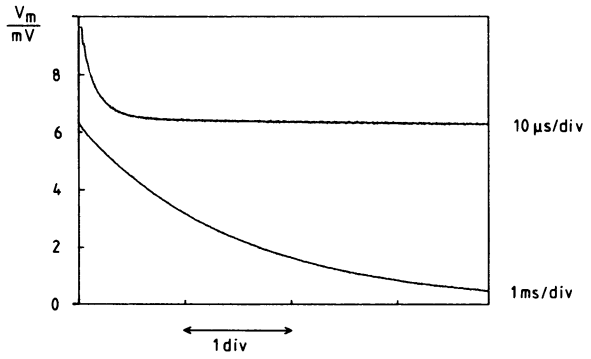

FIGURE 2 Record of a charge-pulse experiment performed on a dioleoyl phosphatidylethanolamine/*n*-decane membrane in a solution containing 1 M NaCl and 3×10^{-8} M TTFPB. At time $t = 0$ the membrane capacitance was charged to a voltage $V_o = 11.13$ mV by a charge pulse of 30 ns duration. The decay of the voltage was recorded with two different sweep times.

eters and k_i, N_t, and τ_m are given by

$$k_i = (a_1/\tau_2 + a_2/\tau_1)/2 \quad (2)$$

$$N_t = 2RTC_m[1/\tau_1 + 1/\tau_2 - 2k_i - 1/(2\tau_1\tau_2k_i)]/F^2k_i \quad (3)$$

$$\tau_m = 2k_i\tau_1\tau_2, \quad (4)$$

where R is the gas constant, T is the absolute temperature, and F is the Faraday constant.

RESULTS

Phosphatidylethanolamine Membranes

Fig. 2 is an experimental record of a charge-pulse experiment taken with the digital oscilloscope from a dioleoyl phosphatidylethanolamine/*n*-decane membrane in the presence of 3×10^{-8} M TTFPB. The values of the membrane potential, $V(t)$, were plotted on a semilogarithmic scale (Fig. 3 *A*) and the amplitude and time constant (V_oa_2, τ_2) of the slower relaxation were determined from a linear least-squares best fit to the data obtained at long times. The logarithm of the difference between the measured voltage and the slow relaxation was then plotted as a function of time (Fig. 3 *B*). The amplitude and time constant of the fast relaxation (V_oa_1, τ_1) were obtained from another linear least-squares best fit. The voltage relaxation of Fig. 3 *B* was very well resolved (time constant, 697 ns). This result clearly indicated that the time resolution of the experimental instrumentation was able to detect voltage relaxations with time constants down to 200 ns.

In all experiments with the negatively charged tetraphenylborate analogues two relaxations were resolved. Whereas the first (fast) purely exponential relaxation process (see Fig. 3 *B*) is coupled with the distribution of lipophilic ions within the membrane and is a function of k_i, the second, slow relaxation process reflects the *RC*-time constant, τ_m (produced either by the membrane resistance or in most cases by a resistor in parallel with the electrodes and the membrane capacitance), and the redistribution of charges. τ_2 was always considerably larger than τ_m because of the adsorbed lipophilic ions (Benz and Conti, 1981; Benz, R., unpublished results). From the parameters of the two relaxation processes the translocation rate constant, k_i, the total surface concentration, N_t, and τ_m were calculated according to Eqs. 2–4 using the specific capacitance of dioleoyl phosphatidylethanolamine/*n*-decane membranes ($C_m = 372$ nF/cm^2; Benz and Janko, 1976). For each set of experimental conditions at least six membranes were used. The standard deviations were usually <15% for k_i and <25% for N_t. The larger variations for N_t are presumably caused by the difficulty in obtaining partition equilibrium which is caused by the large partition coefficients of the lipophilic ions. It has to be noted that the variation of k_i and N_t were not caused by the analysis of the experimental data as Figs. 2 and 3 clearly show because the time constants of the two relaxation processes were always separated by more than one order of magnitude. This means that the deviations mentioned above for the parame-

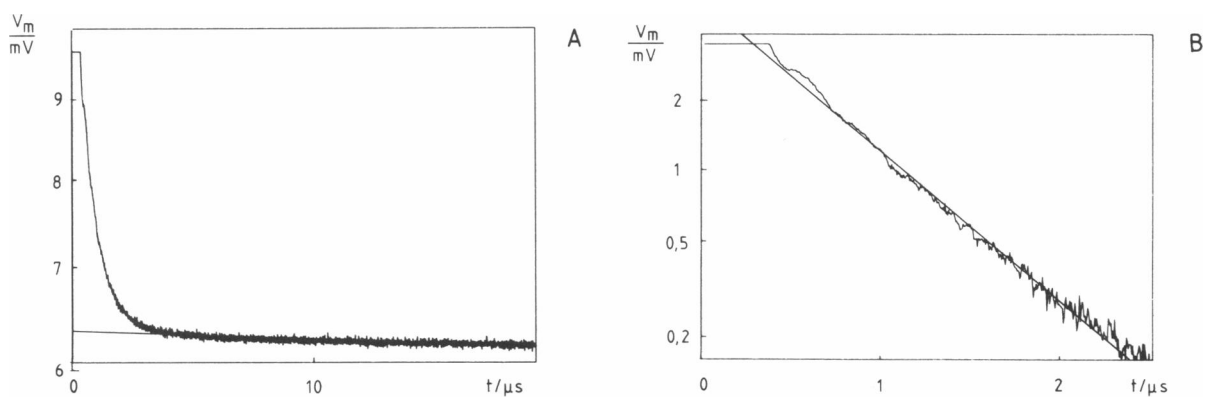

FIGURE 3 Analysis of the experimental data of Fig. 2. The relaxation times τ_1 and τ_2, and amplitudes, V_oa_1 and V_oa_2 (Eq. 1), were obtained from two successive plots, *A* and *B*, as described in the text. (*A*) $V_oa_2 = 6.31$ mV, $\tau_2 = 1.37$ ms. (*B*) $V_oa_1 = 4.82$ mV, $\tau_1 = 697$ ns. From Eqs. 2–4 the following parameters were obtained ($C_m = 372$ nF/cm^2, Benz and Janko, 1976): $N_t = 0.302$ pmol/cm^2, $k_i = 4.07 * 10^5$ 1/s, $\tau_m = 777$ µs.

TABLE I

RESULTS OF CHARGE PULSE EXPERIMENTS PERFORMED ON DIOLEOYL PHOSPHATIDYLETHANOLAMINE/*n*-DECANE MEMBRANES IN THE PRESENCE OF DIFFERENT TETRAPHENYLBORATE ANALOGUES

Compound	$\tau_1/\mu s$	τ_2/s	a_1	$k_i/1/s$	$N_t/pmol/cm^2$	$\beta/10^{-2}$ cm
TPCB	$2.7 * 10^5$	18*	0.68	$6.2 * 10^{-1}$	0.78	1.3
TPB	$1.7 * 10^3$	0.27‡	0.70	$8.8 * 10^1$	0.91	1.5
TEB	$7.4 * 10^2$	0.019§	0.60	$2.7 * 10^2$	0.59	>0.1
TFPB	8.9	0.0021‖	0.63	$2.1 * 10^4$	0.67	1.1
TCPB	2.6	0.0013‖	0.57	$8.1 * 10^4$	0.52	0.87
TTFPB	0.96	0.00093‖	0.27	$3.8 * 10^5$	0.15	0.25

The aqueous phase contained 1 M NaCl and $3 * 10^{-8}$ M lipophilic ions except in the experiments with TEB. TEB was added directly to the aqueous phase, bathing a black membrane in a final concentration of $3 * 10^{-7}$ M and the charge-pulse experiments were performed 1–2 min after equilibration. The other data were obtained 20 min after blackening of the membranes; T = 25°C. *Without shunt resistor. ‡Shunt resistor 10 MΩ. §Shunt resistor 1 MΩ. ‖Shunt resistor 0.1 MΩ.

ters k_i and N_t are not caused by the fit of the experimental data but represent the variations of the individual membranes.

Table I shows the experimental parameters of the charge pulse experiments performed with the different tetraphenylborate analogues on dioleoyl phosphatidylethanolamine membranes. There existed a considerable dependence of the time constant, τ_1, on the type of the analogue, whereas the other parameters were only little influenced by the structure of the lipophilic ions. As described above, these variations of τ_1 were mainly caused by variations of k_i. The translocation rate constant, k_i, increased by a factor of $5 * 10^5$ from the compound TPCB (triphenylcyanoborate, the slowest analogue) to TTFPB (tetrakis(4-trifluoromethylphenyl)borate, with the largest translocation rate constant). This result indicated a considerable influence of the structure of the lipophilic ions on their mobility in the membranes. Even the introduction of a single fluorine onto the phenyl rings led to a 20-fold increase in k_i. On the other hand, the replacement of these fluorines by chlorines had a relatively small influence on the translocation rate constant.

The total concentration of lipophilic ions, N_t, and the partition coefficient, $\beta = N_t/2c$ (with c concentration of the lipophilic ions in the aqueous phase) were only little influenced by the structure of the lipophilic ions. The partition coefficient decreased slightly for increasing translocation rate constants. Only a lower limit could be given for the partition coefficient of TEB (tetraethylborate) because of the rapid hydrolysis of this analogue in the aqueous phase. All the others were stable in the aqueous phase for at least 5 h in the absence of UV light (Benz et al., 1986).

Phosphatidylcholine Membranes

To test whether the strength of the influence of the structure of the lipophilic ions was dependent on the composition of the membrane, similar experiments were performed with dioleoyl phosphatidylcholine/*n*-decane membranes. The results are summarized in Table II. Once again, a considerable influence of the chemical structure of the TPB analogues on the relaxation time constant of the fast process was observed. The translocation rate constant k_i showed a similar structural dependence as in the experiments just described for phosphatidylethanolamine membranes, but k_i was on average about 10 times smaller. This led to the problem that the relaxation parameters for TPCB could not be resolved with the same accuracy as for the other analogues because the initial relaxation was very slow. As before, no partition coefficient for TEB could be derived for phosphatidylcholine membranes because of the

TABLE II

RESULTS OF CHARGE PULSE EXPERIMENTS PERFORMED ON DIOLEOYL PHOSPHATIDYLCHOLINE/*n*-DECANE MEMBRANES IN THE PRESENCE OF DIFFERENT TETRAPHENYLBORATE ANALOGUES

Compound	$\tau_1/\mu s$	τ_2/s	a_1	$k_i/1/s$	$N_t/pmol/cm^2$	$\beta/10^{-2}$ cm
TPCB	$1.3 * 10^6$	25*	0.82	$8.0 * 10^{-2}$	1.2	2.0
TPB	$1.4 * 10^4$	0.23‡	0.73	9.5	1.0	1.7
TEB	2.7×10^3	0.41‡	0.82	$3.3 * 10^1$	1.8	>0.3
TFPB	87	0.0021§	0.67	$1.9 * 10^3$	0.79	1.3
TCPB	26	0.0019§	0.58	$7.9 * 10^3$	0.55	0.92
TTFPB	7.3	0.00086§	0.35	$4.5 * 10^4$	0.21	0.35

The aqueous phase contained 1 M NaCl and $3 * 10^{-8}$ M lipophilic ions except in the experiments with TEP. TEP was added directly to the aqueous phase bathing a black membrane in a final concentration of $3 * 10^{-7}$ M and the charge-pulse experiments were performed 1–2 min after equilibration. The other experimental data were obtained 20 min after blackening of the membranes; T = 25°C. *Without shunt resistor. ‡Shunt resistor 10 MΩ. §Shunt resistor 0.1 MΩ.

TABLE III
TEMPERATURE DEPENDENCE OF THE KINETIC PARAMETERS OF THE TETRAPHENYLBORATE ANALOGUES IN MEMBRANES MADE OF TWO DIFFERENT LIPIDS

T/°C	$\tau_1/\mu s$	τ_2/s	a_1	$k_i/1/s$	$N_t/pmol/cm^2$	$\beta/10^{-2}$ cm
Dioleoyl phosphatidylethanolamine						
TPCB						
5	$7.4 * 10^5$	25*	0.75	$1.8 * 10^{-1}$	1.0	1.7
45	$1.5 * 10^5$	18*	0.64	1.2	0.66	1.1
TTFPB						
5	3.9	0.0010‡	0.35	$8.4 * 10^4$	0.21	0.35
45	0.49	0.00076‡	0.26	$7.6 * 10^5$	0.14	0.23
Dioleoyl phosphatidylcholine						
TPB						
5	$3.7 * 10^4$	0.30§	0.75	3.5	1.1	1.8
45	$6.0 * 10^3$	0.19§	0.63	$3.1 * 10^1$	0.66	1.1
TFPB						
5	290	0.0023‡	0.68	$5.5 * 10^2$	0.84	1.4
45	52	0.0018‡	0.61	$3.8 * 10^3$	0.61	1.0
TCPB						
5	55	0.0019‡	0.66	$3.1 * 10^3$	0.78	1.3
45	13	0.0017‡	0.58	$1.8 * 10^4$	0.54	0.90
TTFPB						
5	18	0.0010‡	0.38	$1.7 * 10^4$	0.25	0.41
45	3.9	0.0011‡	0.31	$9.3 * 10^4$	0.17	0.28

The aqueous phase contained 1 M NaCl and $3 * 10^{-8}$ M of the lipophilic ions. The experiments were performed 20 min after the blackening of the membranes. *Without shunt resistor. ‡Shunt resistor 0.1 MΩ. §Shunt resistor 10 MΩ.

rapid aqueous hydrolysis of this compound in the aqueous phase.

Temperature Dependence

The temperature dependence of the relaxations modified by some of the tetraphenylborate analogues was studied in separate experiments. Table III shows the results with TPCB, TPB, TFPB, TCPB, and TTFPB. The partition coefficient, β, decreased slightly with increasing temperature, whereas the translocation rate constant, k_i, strongly increased with increasing temperature. The temperature dependence of k_i was used to calculate the activation energy using Arrhenius plots. Fig. 4 shows Arrhenius plots of the translocation rate constants of TTFPB in phosphatidylethanolamine and phosphatidylcholine membranes. E_a was in these cases ~41 and 32 kJ/mol, respectively. The activation energies of the other systems were very similar, and they are shown in Table V.

DISCUSSION

In this work, charge pulse experiments were performed with structural analogues of tetraphenylborate (TPB) on two different types of artificial lipid bilayer membranes. The results of the measurements are consistent with the assumption that the mechanism proposed for the transport of lipophilic ions (Ketterer et al., 1971; Benz et al., 1976; Benz and Conti, 1981) is also valid for the transport of these analogues. The analysis of the experimental data in terms of the model yields the translocation rate constant, k_i, and the partition coefficient, β. The values of the adsorption and the desorption rate constants have to be left open because of slow aqueous diffusion of the lipophilic ions near the membrane–water interface (Benz et al., 1976). The partition coefficients, β, were similar for all TPB analogues and did not vary much. On the other hand, it is also evident from a comparison of the data of Tables I and II that analogues with larger translocation rate constants have smaller partition coefficients. This may be caused by small shifts of the adsorption planes as a

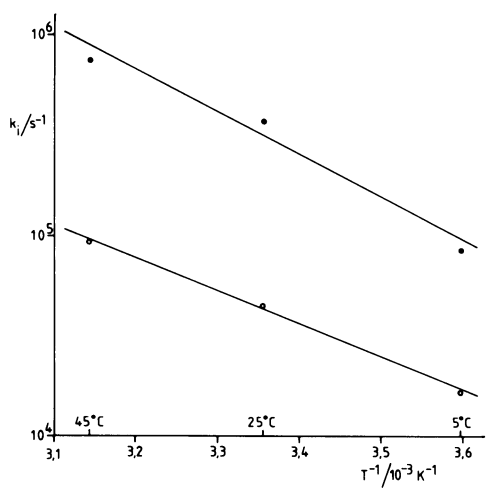

FIGURE 4 Arrhenius plots of the translocation rate constants of TTFPB in phosphatidylethanolamine (*solid circles*) and phosphatidylcholine (*open circles*) membranes. The straight lines correspond to activation energies of 41 and 32 kJ/mol, respectively.

TABLE IV
THERMODYNAMIC PARAMETERS OF THE ADSORPTION OF THE TETRAPHENYLBORATE ANALOGUES TO LIPID BILAYER MEMBRANES FOR T = 25°C

Compound	$K/10^4$	ΔG/kJmol^{-1}	ΔH/kJmol^{-1}	$T\Delta S$/kJmol^{-1}
Dioleoyl phospatidylethanolamine				
TPCB	26	−30.9	−8.0	22.9
TPB	30	−31.2	—	—
TEB	>2.0	<−24.5	—	—
TFPB	22	−30.5	—	—
TCPB	17	−29.9	—	—
TTFPB	5.0	−26.8	−7.7	19.1
Dioleoyl phosphatidylcholine				
TPCB	40	−31.9	—	—
TPB	34	−31.5	−7.4	24.1
TEB	>6.0	<−27.2	—	—
TFPB	26	−30.9	−6.2	24.7
TCPB	18	−30.0	−6.9	23.1
TTFPB	7.0	−27.6	−7.0	20.6
TPP*	0.11	−17.1	16.3	33.4

The parameters were calculated from the data given in Tables I–III using Eqs. 5–7. *Values for TPP (T = 20°C) were taken from Flewelling and Hubbell, 1986*a*.

TABLE V
FREE ENERGY DIFFERENCES AND ACTIVATION ENERGY FOR THE MOVEMENT OF THE TPB ANALOGUES ACROSS MEMBRANES

Compound	$\Delta G°$/kJmol^{-1}	$\Delta G° - \Delta G$/kJmol^{-1}	E_a/kJmol^{-1}
Dioleoyl phosphatidylethanolamine			
TPCB	13.7	44.6	35
TPB	1.0	32.2	—
TEB	<4.9	29.4	—
TFPB	−11.6	18.9	—
TCPB	−14.5	15.4	—
TTFPB	−15.3	11.4	41
Dioleoyl phosphatidylcholine			
TPCB	17.0	47.6	—
TPB	6.2	37.7	40
TEB	<7.4	34.6	—
TFPB	−6.2	24.7	36
TCPB	−8.9	21.1	32
TTFPB	−10.8	16.8	32

The free energies were calculated from the data of Tables I, II, and IV using Eq. 9. The activation energies were calculated from the results given in Tables I–III.

consequence of higher solubilities of certain analogues in the membrane interior.

The partition coefficient, β, between the aqueous phase and the adsorption site may be used to calculate the binding constant, K, and the free energy, ΔG, of adsorption according to

$$K = \beta/l_a \tag{5}$$

$$\Delta G = -RT\ln K, \tag{6}$$

where l_a is equal to the thickness of the adsorption plane in the membrane (i.e., $\beta/l_a = N_t/2N_a$ with N_a concentration in mol/cm^2 of the lipophilic ions at the aqueous side of the membrane–water interface). It is assumed that l_a is temperature-independent and approximately equal to 0.5 nm (corresponding to the thickness of the polar headgroup layer). ΔG was calculated for the different TPB-analogues and the two different types of membranes from the data of β given in Tables I and II. The results are summarized in Table IV. The free energy of adsorption, ΔG, of the TPB-analogues may be compared with the free energy change, ΔG^*, associated with the transfer of tetraphenylmethane (the neutral equivalent of tetraphenylborate) from water into a hydrocarbon. The hydrocarbon/water partition coefficient of tetraphenylmethane is approximately 10^6 (Andersen and Fuchs, 1975). Thus, ΔG^* is ~ -34 kJ/mol. The difference between ΔG and ΔG^* is expected from theory because ΔG contains, besides ΔG^*, two additional energy contributions, that of the image force (which tends to increase ΔG) and that of the large positive dipole potential of the membranes (which tends to decrease ΔG). The large positive dipole potential (~215–240 mV for both phosphatidylcholine and phosphatidylethanolamine membranes; Pickar and Benz, 1978; Flewelling and Hubbell, 1986*b*) of lipid bilayer membranes is responsible for the large difference between the partition coefficients for TPB and for its positively charged analogues TPP (tetraphenylphosphonium) and tetraphenylarsonium (Liberman and Topaly, 1969; Pickar and Benz, 1978; Flewelling and Hubbell, 1986*b*).

The translocation rate constants of the different compounds were about a factor of 9 larger for phosphatidylethanolamine membranes as compared with phosphatidylcholine membranes. On the other hand, the partition coefficient, β, was on average a factor of 1.5 smaller for phosphatidylethanolamine. This means that the lipophilic ions experience on their way from the aqueous phase to the center of the membranes a potential difference of 50 mV between both types of membranes. This potential difference is in agreement with earlier studies (Benz and Gisin, 1978; Pickar and Benz, 1978; Flewelling and Hubbell, 1986*b*) and may be caused by small structural differences of the polar headgroups and of the packing of both lipids.

The temperature dependence of the adsorption of lipophilic ions may be used for the calculation of the enthalpy of adsorption, ΔH, according to

$$\Delta H = \Delta G + T\Delta S = RT^2(d\ln K/dT). \tag{7}$$

The results for ΔH and $T\Delta S$ are also given in Table V. ΔH was always negative, whereas $T\Delta S$ was in all cases positive. This means that the adsorption of the lipophilic ions is partly entropy driven. Similar conclusions were already drawn by Bruner (1975) and Benz et al. (1976) for dipicrylamine and by Flewelling and Hubbell (1986*a*) for TPP, the positively charged analogue of TPB. The basic

difference (besides the smaller binding constant) between positively and negatively charged lipophilic ions consisted in a large difference of the enthalpy of adsorption, ΔH. Whereas ΔH was negative for TPB and its analogues (~ -7 kJ/mol; compare with Table IV) it was positive (~ 16 kJ/mol) for TPP (Flewelling and Hubbell, 1986*a*). Hydrostatic pressure leads to an increase of the partition coefficient of lipophilic ions (Benz and Conti, 1986), i.e., the adsorption coefficient has a negative apparent volume of adsorption. This finding is consistent with the conclusions just drawn that the adsorption of lipophilic ions to lipid bilayer membranes is partly entropy driven.

The dramatic influence of the structure of the TPB analogues on the translocation rate constant may be explained by an increase of the effective radii (i.e., the smearage of the charge across larger spheres) of these compounds as we ascend the series TPCB to TTFPB. This increase of the effective radius apparently results in a decrease of the central barrier in the membrane. The change of the barrier height could be calculated from the change of the free energy difference, ΔG°, between the aqueous phase and the center of the membrane which is given by

$$\Delta G^\circ = -RT\, ln(RTG_o, G_{o,o}d/F^2Dc), \qquad (8)$$

where $G_{o,o}$ is the initial ohmic conductance at zero voltage, $D = 10^{-7}$ cm^2/s is the diffusion coefficient within the membrane, and $d = 5$ nm is the membrane thickness (Benz and Janko, 1976). Introducing the expression for $G_{o,o}$ (Ketterer et al., 1971) Eq. 8 becomes

$$\Delta G^\circ = -RT\, ln(\beta k_i d/D). \qquad (9)$$

Table V shows the values for ΔG° and for the difference between ΔG° and ΔG, i.e., the absolute height of the barrier in the middle of the membrane (with respect to the depths of the interfacial wells). In the series from TPCB to TTFPB the free energy difference ΔG° decreased by 29 kJ/mol (phosphatidylethanolamine membranes) and by ~ 28 kJ/mol (phosphatidylcholine membranes). The absolute height of the central barrier ($\Delta G^\circ - \Delta G$) decreased simultaneously by ~ 33 kJ/mol and ~ 31 kJ/mol, respectively.

Fig. 5 shows the free energy profiles for TPCB and TTFPB in ethanolamine membranes. Both profiles are only approximate values because a value for the diffusion coefficient of the lipophilic ions within the membranes had to be assumed for the calculation of ΔG°. Consider first the free energy profile for TPCB. It is not clear if this compound and TPB experience a similar interfacial barrier (Jordan and Stark, 1979). The drop of the free energy, ΔG, as referred to the aqueous phase is ~ -31 kJ/mol (~ -7.4 kcal/mol). The free energy difference between the aqueous phase and the center of the membrane is ~ 14 kJ/mol (3.4 kcal/mol), which means that the central barrier (with

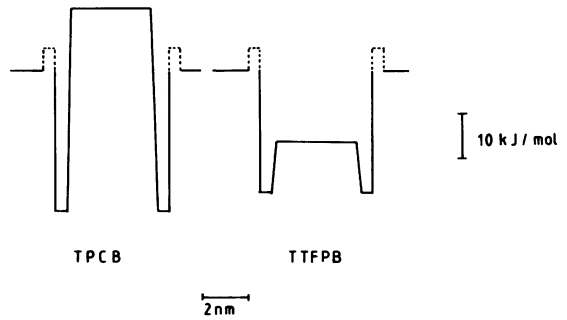

FIGURE 5 Profile of the free energy, ΔG, of TPCP and TTFPB in a phosphatidylethanolamine/*n*-decane membrane. See text for details.

respect to the depth of the adsorption wells) is almost 45 kJ/mol (10.7 kcal/mol) for TPCB. The exact barrier shapes for this lipophilic ion and for TTFPB are not known. For the sketch of the free energy profiles in Fig. 5 it was assumed that the shapes are very similar to that of TPB, which means that they can be adequately modeled as a trapezoid with a shorter side which has a length of about 0.9 of the base (Andersen and Fuchs, 1975, Andersen et al., 1978). A comparison between the free energy profiles for TPCB and TTFPB shows that the adsorption wells for TTFPB are somewhat shallower. The considerably smaller height of the central barrier of the latter lipophilic ion is more dramatic because it had only a magnitude of 11.4 kJ/mol (2.7 kcal/mol), compared with 44.6 kJ/mol (10.7 kcal/mol) for that of TPCB. It has to be noted that the central barrier for TTFPB is below the free energy of the water phase (see Fig. 5). This means that this lipophilic ion is more soluble in the membrane interior than in the aqueous phase. Similar results have been found for the anionic forms of the uncouplers FCCP (carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone) (Benz and McLaughlin, 1983) and S-13 (5-chloro-3-tert-butyl-2′-chloro-4′nitrosalicylanilide) (Kasianowicz et al., 1987) in phosphatidylcholine/chlorodecane membranes.

As mentioned already, this dramatic decrease of ΔG° from TPCB to TTFPB is most likely caused by the smearage of the negative charge across a larger sphere, i.e., the increase of the effective diameter of most TPB analogues as compared with TPB. A quantitative description of this effect is somewhat difficult to obtain. The reason for this is that ΔG° contains several different contributions.

$$\Delta G^\circ = \Delta W_E + \Delta W_D + \Delta G^*, \qquad (10)$$

where ΔW_E is the contribution of the Born energy and of the image force and ΔW_D is the contribution of the dipole potential (Pickar and Benz, 1978; Flewelling and Hubbell, 1986*b*). ΔG^* is the neutral contribution. ΔW_E is given by (Parsegian, 1969).

$$\Delta W_E = \frac{e^2}{8\pi\epsilon_o r}\left(\frac{1}{\epsilon_m} - \frac{1}{\epsilon_w}\right) - \frac{e^2}{4\pi\epsilon_o\epsilon_m d}\, ln\left(\frac{2\epsilon_w}{\epsilon_w + \epsilon_m}\right). \qquad (11)$$

ΔW_E is the electrostatic energy (in units of kT) which must be overcome by an ion of radius r and charge e going from the aqueous phase (relative dielectric constant ϵ_w) into the center of a lipid bilayer (relative dielectric constant ϵ_m and thickness d). The first term is dependent on the ion radius. The second term is only dependent on the bilayer thickness and is identical for all TPB analogues.

From the other contributions to ΔG° only ΔG^* could be dependent on the structure of the lipophilic ions because this term contains contributions such as van der Waals and hydrophobic interactions. If it is assumed that the change of ΔG^* is negligible for the different analogues as compared with the change of the electrostatic energy, the effective radius r^* of the TPB analogues may be calculated from the difference of the free energies $\Delta\Delta G^\circ$ according to

$$\Delta\Delta G^\circ = A(1/r^* - 1/r_{TPB}), \qquad (12)$$

where $A = 3.21*10^{-3}$ Jcm/mol. Table VI shows the effective radii of the different TPB analogues calculated from Eq. 12 assuming that the radius of TPB is approximately given by its van der Waals radius ($r_{TPB} = 0.42$ nm). The effective radii range from ~0.36 nm (TPCB) to ~0.58 nm (TTFPB). The least precise value for r^* is presumably that of TEB where ΔG^* has presumably a much larger contribution to ΔG° than for the other TPB analogues. It is interesting to note that excellent agreement exists between the effective radii calculated from the data from phosphatidylethanolamine and from phosphatidylcholine membranes.

The effective radii of the TPB analogues follow to a certain extent the shift of the electron distribution by the various substituents with the exception of the fluoro compound because the fluoro-substituent should be more negative than the chloro-analogue, but TCPB has definitely a larger effective radius than TCPB (see Table VI). On the other hand, it seems to be very difficult to derive an effective radius from the electron distribution. A more meaningful comparison may be obtained from molecular models of the different compounds. In fact, the van der Waals radii of TFPB, TCPB, and TTFPB as derived from CPK Precision Molecular Models (Ealing Co., Watford, UK) are ~0.50, 0.54, and 0.60 nm, respectively, which is close to the radii calculated from Eq. 12. A similar comparison is not possible for TPCB because of its asymmetry. On the other hand, it has to be noted that the comparison of the data of Table VI with CPK models is not convincing at all because the ionic radii of the TPB analogues have most likely only little to do with van der Waals radii.

TABLE VI
EFFECTIVE RADII OF THE DIFFERENT TPB ANALOGUES

Analogue	r^*/nm	
	Phosphatidylethanolamine	Phosphatidylcholine
TPCB	0.361	0.372
TPB*	0.420	0.420
TEB	0.436	0.438
TFPB	0.508	0.506
TCPB	0.535	0.535
TTFPB	0.577	0.578

The effective radii were calculated using Eq. 12 and the values for ΔG° given in Table V. *The effective radius of TPB was assumed to be equal to its van der Waals radius.

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REFERENCES

Andersen, O. S., and M. Fuchs. 1975. Potential energy barriers to ion barriers to ion transport within lipid bilayers. Studies with tetraphenylborate. *Biophys. J.* 15:795–830.

Andersen, O. S., S. Feldberg, H. Nakadomari, S. Levy, and S. McLaughlin. 1978. Electrostatic potentials associated with the absorption of tetraphenylborate into lipid bilayer membranes. *In* Membrane Transport Processes. Vol. 2. D. C. Tosteson, Y. A. Ovchinnikov, and R. Latorre, editors. Raven Press, New York. 325–334.

Benz, R., and F. Conti. 1981. Structure of the squid axon membrane as derived from charge-pulse relaxation studies in the presence of absorbed lipophilic ions. *J. Membr. Biol.* 59:91–104.

Benz, R., and F. Conti. 1986. Effects of hydrostatic pressure on lipid bilayer membranes. I. Influence on membrane thickness and activation volumes of lipophilic transport. *Biophys. J.* 50:91–98.

Benz, R., and D. Cros. 1978. Influence of sterols on ion transport through lipid bilayer membranes. *Biochim. Biophys. Acta.* 506:265–280.

Benz, R., and B. F. Gisin. 1978. Influence of membrane structure on ion transport through lipid bilayer membranes. *J. Membr. Biol.* 40:293–314.

Benz, R., and K. Jenko. 1976. Voltage-induced capacitance relaxation of lipid bilayer membranes: effects of membrane composition. *Biochim. Biophys. Acta.* 455:721-738.

Benz, R., and P. Läuger. 1977. Transport kinetics of dipicrylamine through lipid bilayer membranes. Effects of membrane structure. *Biochim. Biophys. Acta.* 468:245–258.

Benz, R., and S. McLaughlin. 1983. The molecular mechanism of action of the proton ionophore FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone). *Biophys. J.* 41:381–398.

Benz, R., and W. Nonner. 1981. Structure of the axolemma of frog myelinated nerve. Relaxation experiments with a lipophilic probe ion. *J. Membr. Biol.* 49:127–134.

Benz, R., and U. Zimmermann. 1983. Evidence for the presence of mobile charges in the cell membrane of *Valonia utricularis*. *Biophys. J.* 43:13–26.

Benz, R., F. Conti, and R. Fioraventi. 1984. Extrinsic charge movement in the squid axon membrane: effect of pressure and temperature. *Eur. Biophys. J.* 11:51–59.

Benz, R., R. Elbert, W. Prass, and H. Ringsdorf. 1986. Polymerization in black lipid membranes. Influence on ion transport. *Eur. Biophys. J.* 14:83–92.

Benz, R., P. Läuger, and K. Janko. 1976. Transport kinetics of hydrophobic ions in lipid bilayer membranes. Charge-pulse relaxation studies. *Biochim. Biophys. Acta.* 455:701–720.

Bruner, L. J. 1975. The interaction of hydrophobic ions with lipid bilayer membranes. *J. Membr. Biol.* 22:125–141.

Dilger, J. P., and R. Benz. 1985. Optical and electrical properties of thin monoolein membranes. *J. Membr. Biol.* 85:181–189.

Flewelling, R. F., and W. L. Hubbell. 1986*a*. Hydrophobic ion interactions with membranes. Thermodynamic analysis of tetraphenylphosphonium binding to vesicles. *Biophys. J.* 49:531–540.

Flewelling, R. F., and W. L. Hubbell. 1986*b*. The membrane dipole potential in a total membrane potential model. Applications to hydrophobic ion interactions with membranes. *Biophys. J.* 49:541–552.

Jordan, P. C., and G. Stark. 1979. Kinetics of transport of hydrophobic ions through lipid membranes including diffusion polarization in the aqueous phase. *Biophys. Chem.* 10:273–287.

Kasianowicz, J., R. Benz, and S. McLaughlin. 1987. How do protons cross the membrane-solution interface? Kinetic studies on bilayer membranes exposed to the protonophore S-13 (5-chloro-3-tert-butyl-2′-chloro-4′nitrosalicylanilide). *J. Membr. Biol.* 95:73–89.

Ketterer, B., B. Neumke, and P. Läuger. 1971. Transport mechanism of hydrophobic ions through lipid bilayer membranes. *J. Membr. Biol.* 5:225–245.

Liberman, E. A., and V. P. Topaly. 1969. Permeability of bimolecular phospholipid membranes for fat-soluble ions. *Biophysics.* 14:477–487.

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* 9:71–144.

Parsegian, A. 1969. Energy of an ion crossing a low dielectric membrane: solutions for four relevant electrostatic problems. *Nature (Lond.).* 221:844–846.

Pickar, A. D., and R. Benz. 1978. Transport of oppositely charged lipophilic probe ions in lipid bilayer membranes having various structures. *J. Membr. Biol.* 44:353–376.

Szabo, G. 1974. Dual mechanism for the action of cholesterol on membrane permeability. *Nature (Lond.).* 252:47–49.

Wulf, J., R. Benz, and W. G. Pohl. 1977. Properties of bilayer membranes in the presence of dipicrylamine. *Biochim. Biophys. Acta.* 465:429–442.