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THE BLOCKAGE OF THE ELECTRICAL CONDUCTANCE IN A PORE-CONTAINING MEMBRANE BY THE *n*-ALKANES

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Summary

1. In monooelein bilayers made highly conducting by the addition of a fixed amount of *o*-pyromellitylgramicidin, the membrane conductance has been shown to be strongly dependent on the chain length of the *n*-alkane with which the membrane is in equilibrium. Thus for *n*-hexadecane, the conductance is larger by approx. 10^4 times than it is for *n*-octane. This result is independent of whether the polypeptide is introduced via the aqueous or lipid phases.

2. The observed conductance variations have been accounted for in terms of a mechanism (outlined in earlier publications) which is based on the thickness and tension changes produced in bilayers by the adsorption of *n*-alkanes. Essentially quantitative agreement between theory and experiment is found.

Introduction

Gramicidin A is known to form ion-conducting pores in 'black' lipid membranes [1—4]. These pores are believed to arise from the dimerization of gramicidin monomers [5,6] which are effectively irreversibly adsorbed onto the membrane surfaces [1,2]. When black membranes are formed from the same lipid, but using different chain length alkanes (e.g. *n*-decane and *n*-hexadecane) it has been noted that similar additions of gramicidin to the systems produce widely different levels of conductance [7]. The conductance of the unit channel of gramicidin is, on the other hand, independent of the alkane chain length [1,8]. It must be concluded, therefore, that the alkanes affect the number of channels conducting at any one time or, in other words, that they affect the constant of equilibrium between the non-conducting monomers and conducting dimers.

The mechanism of the alkane effect is not yet entirely clear. However, one

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property of phospholipid and monoglyceride black films which depends strongly on the alkane used is the electrical capacity per unit area, which tends to be larger for the longer chain homologues [9—13]. Since the area per molecule of the lipid in the membranes is not a function of the alkane chain length [9,10] it appears that the alkanes partition predominantly into the central part of the bilayer [9]. As a consequence, the thickness of the lipophilic region must change and this is believed to account for the observed capacity variations [9—14]. Furthermore, since the thicknesses of the membranes in question all exceed the length attributed to the gramicidin pore and since, in the presence of alkanes, the bilayers have appreciable tensions, it is to be expected that the distortion or dimpling of the membrane necessary for open pore formation would reduce the stability of the conducting structures. The idea that membrane thickness is a factor in regulating gramicidin conductance has also arisen from the study of current-voltage relations in monoolein-alkane systems. Thus, the relatively thick bilayers (formed from lower alkanes) are readily compressible in electric fields and show conductances which increase rapidly with potential whereas the thinner, less compressible, bilayers give more nearly ohmic behaviour [2,7,15—17].

In order to test more thoroughly the validity of the thickness-tension mechanism for gramicidin conduction it is desirable that quantities of the polypeptide may be added reproducibly to different membranes. Owing to its insolubility in both aqueous and alkane solutions, this is very hard to achieve with gramicidin itself. However, the recent synthesis of the water-soluble derivative *o*-pyromellitylgramicidin has helped greatly to overcome this difficulty [18]. Like its parent compound, *o*-pyromellitylgramicidin forms pores across the bilayer by a second order process [18]. Black lipid films of monooleic and the alkanes heptane to hexadecane are well characterized as to thickness and tension [10, 19]. The conductances of these membranes have therefore been studied after controlled additions of *o*-pyromellitylgramicidin from both the aqueous phases and from the film-forming lipid solutions.

Materials and Methods

o-Pyromellitylgramicidin was synthesized from commercial (Koch-Light) gramicidin in the way described by Apell et al. [18]. The purification of the raw product was carried out by thin-layer chromatography using silica gel 60 plates (Merck) with chloroform/methanol/water (65 : 25 : 4, v/v). This yielded a very prominent spot of R_F 15—20, which Apell et al. showed to be the *o*-pyromellityl derivative, and a weaker spot of R_F 75 which corresponded to unreacted gramidicin. Spectroscopic and hydrolytic tests of the product also gave results in agreement with those of Apell et al. Monoolein was obtained from Nu-Chek Prep, Elysian, Minn., U.S.A. The alkanes were puriss specimens from Koch-Light Ltd. and were further purified by passage through alumina columns. Solvents and inorganic materials were of analytical reagent grade and the water was twice distilled.

Monoolein 'black' films were made across a hole in a vertical polytetrafluorethylene (PTFE) septum by the syringe method described previously [14]. The membrane conductance both at high and single channel levels was measured as

in previous investigations [14]; the potential applied in the measurement of high conductance levels was 10 mV.

In the first series of experiments in which the *o*-pyromellitylgramicidin was added via the aqueous phase, a solution of 10 mM LiCl was used. *o*-Pyromellitylgramicidin concentrations of between 1.6 and 33 nM were employed and, between measurements, the cell was emptied by suction and washed in polypeptide-free solution. When a membrane was formed in the presence of the *o*-pyromellitylgramicidin, its conductance and area were monitored as a function of time. The films examined in this series were of monoolein (6 mM) in the alkanes *n*-heptane to *n*-tetradecane. For the lower alkanes special attention was given to equilibrating the lipid and aqueous solutions before commencing an experiment.

In the second series of experiments *o*-pyromellitylgramicidin was introduced via the lipid phase. To 2 ml of the 6 mM monoolein-alkane solution was added 25 μl of a $5 \cdot 10^{-4}$ M methanolic solution of the polypeptide. This largely immiscible mixture was shaken by hand for 10 min and then centrifuged to separate the methanol from the hydrocarbon. 1 ml of the supernatant alkane solution was then used to make films. A variation of this method, in which a monoolein concentration of 18 mM was used, was also tried. The aqueous phase in these experiments was 0.1 M NaCl. Alkanes from *n*-heptane to *n*-hexadecane were used.

The temperature in all instances was between 21 and 24°C.

Results

When *o*-pyromellitylgramicidin was added via the aqueous phase to a monoolein-decane black film the membrane conductances per unit area observed for different polypeptide concentrations were as shown in Fig. 1. Although it appears that the conductances are approaching a limiting value, they were never found to become completely constant and continued to creep upwards even after 30 min. However, the reproducibility of the conductances became progressively poorer at longer times and only the data obtained in the first few minutes were considered suitable for quantitative analysis. In order to compare the effect of different polypeptide concentrations on a given type of film the conductances were noted at a given time (4 min) after film formation. Other alkanes behaved in much the same way as did the decane systems. The mean conductances per unit area after 4 min for a given *o*-pyromellitylgramicidin concentration are shown in Fig. 2 for the various alkanes used in the film formation.

When the black membranes were formed with monoolein (6 mM)-alkane solutions which had been shaken with methanolic *o*-pyromellitylgramicidin they acquired a relatively large conductance per unit area which usually became constant after 10—20 min. Some of the conductance data for different alkanes are shown in Fig. 3. As for the experiments in which the *o*-pyromellitylgramicidin was added via the aqueous phase, much higher conductances were obtained for the longer chain length alkanes. All the steady state conductances for the lipid phase addition of *o*-pyromellitylgramicidin are shown, as a function of the alkane used, in Fig. 4. Included in this figure are data for heptane to

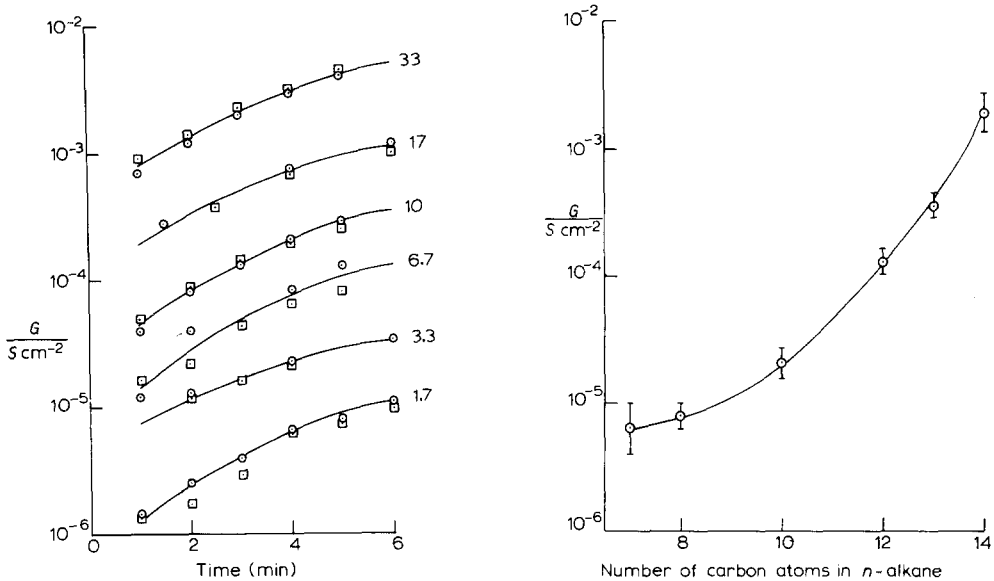

Fig. 1. Membrane conductance per unit area as a function of time after formation in the presence of aqueous *o*-pyromellitylgramicidin. The aqueous concentration of *o*-pyromellitylgramicidin (nM) is shown to the right of each curve. The symbols ⊙ and ⊡ indicate different membranes at the same concentration. The membrane-forming solution was 6 mM monoolein in *n*-decane and the aqueous phase was 10 mM LiCl.

Fig. 2. Mean membrane conductance per unit area (2—4 experiments) 4 min after formation as a function of the chain length of the *n*-alkane solvent in equilibrium with the membrane. All membranes were made in the presence of 3.3 nM *o*-pyromellitylgramicidin in the aqueous phase, which was 10 mM LiCl. The spread of the results is indicated by the error bars.

dodecane, where the monoolein concentration was raised to 18 mM. The effect of the alkane chain length is again seen although the conductances are, in this instance, higher. It appears that the monoolein is at least partially responsible for the partitioning of the *o*-pyromellitylgramicidin into the alkane solutions.

An examination of the unit channel conductances of the *o*-pyromellityl-gramicidin was carried out for monoolein-decane and monoolein-hexadecane membranes. Instead of a single peak in the amplitude histogram, as for pure gramicidin, four peaks were discernible. It would appear, therefore, that despite the chromatographic purity of the *o*-pyromellitylgramicidin, it contained some unreacted gramicidin and that, as Apell et al. [18] inferred, this gives rise to channels of pure gramicidin, pure *o*-pyromellitylgramicidin and asymmetric hybrid channels whose conductance depends on the polarity of the applied potential. The unit channels observed had the same conductances in hexa-decane- as in decane-monoolein membranes and, where comparable, were in agreement with those reported previously [18]. No evidence was found that either the ratios of the probabilities of occurrence or of the mean durations of these various channels differed significantly as between the hexadecane and decane membranes.

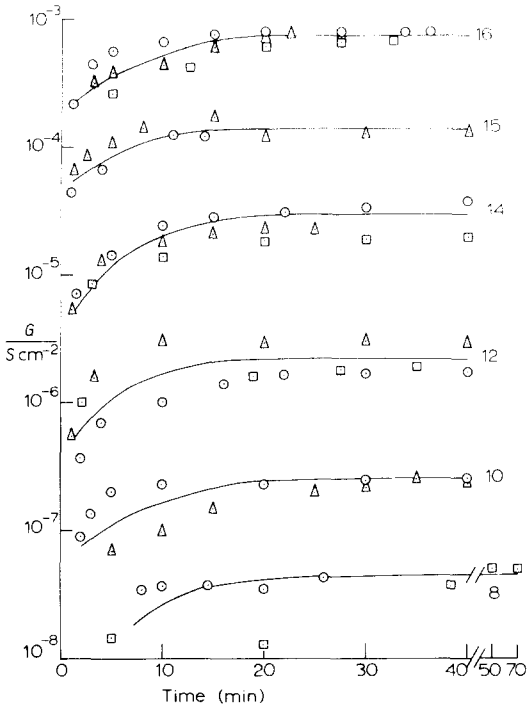

Fig. 3. Membrane conductance per unit area as a function of time after formation. The number to the right of each curve indicates the number of carbon atoms in the *n*-alkane solvent in the 6 mM solution of monoolein used to make the membranes. Symbols ○, △ and □ indicate different membranes containing the same solvent. *o*-Pyromellitylgramicidin was present in the lipid phase as described in the text. The aqueous phase was 0.1 M NaCl.

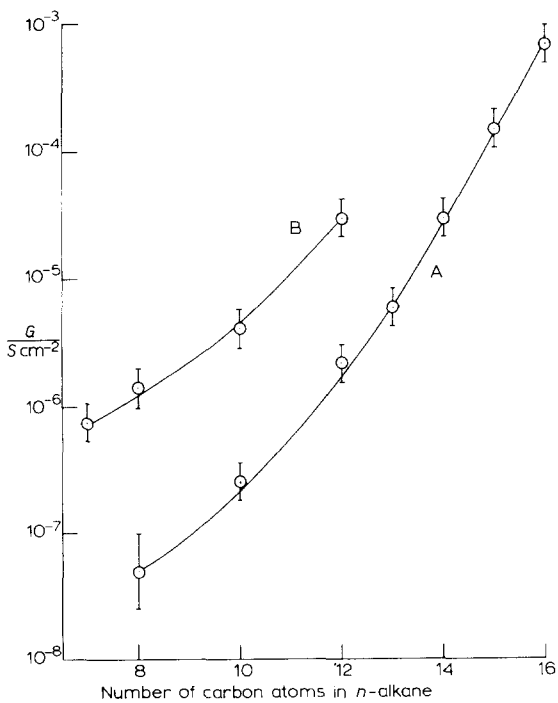

Fig. 4. Mean steady-state membrane conductance per unit area as a function of the chain length of the *n*-alkane solvent in equilibrium with the film. *o*-Pyromellitylgramicidin was added to these membranes via the lipid phase, as described in the text. Line A was obtained using 6 mM monoolein in alkane, line B using 18 mM. Each experiment was repeated three or four times; the error bars indicate the spread of the

Discussion

The adsorption of the o-pyromellitylgramicidin

The inconveniently long times required to reach adsorption equilibrium when the *o*-pyromellitylgramicidin was added to the aqueous phases might have arisen from an effectively irreversible interaction of the polypeptide with the membranes and other surfaces in the cell * or, since gramicidin often behaves in this manner, they might have reflected the presence of the small amounts of this substance which were known to be present from the single channel measurements. The distinction between these possibilities is not important for the present discussion and it is sufficient to note that the conductances at relatively short times were reproducible. A square law relationship between conductance and the nominal aqueous *o*-pyromellitylgramicidin concentration was found, in agreement with the work of Apell et al. [18].

The introduction of the *o*-pyromellitylgramicidin into the lipid solutions by equilibrating them with a methanolic solution of the polypeptide appears to be possible only because of the presence of the monoolein. Thus, not only does the conductance of the membranes increase when a higher concentration of monoolein is used but also, if the monoolein is added to the alkane after contact with the methanolic polypeptide, little or no conductance is produced. Since the two concentrations of monoolein employed (6 and 18 mM) are well above the critical micelle concentration of the lipid in alkanes (approx. 1.5 mM [9]), the *o*-pyromellitylgramicidin is evidently solubilized in the lipid micelles, as might be expected for a surface-active material.

A crucial requirement for the analysis presented in the next section is that the adsorption of the *o*-pyromellitylgramicidin monomers should not be a function of the alkane used in the film-forming lipid solution. While there seems to be no really rigorous means of demonstrating that this is correct there are various reasons for supposing that it is a reasonable assumption. Thus, where adsorption occurs from the aqueous phases the alkanes neither dissolve in these phases to any great extent, nor do they appear to have much effect on the outer regions of the lipid region of the membranes [9,21]. Furthermore, when adsorption occurs from the lipid, the *o*-pyromellitylgramicidin is apparently supplied from the micelles of monoolein and there is no evidence that these differ greatly in their properties from one alkane to another [20]. The most convincing argument, however, is that the dependence of the membrane conductance on the alkane chain length is the same regardless of the route by which the polypeptide is introduced. The only process common to these routes is pore formation from adsorbed monomers.

The dependence of the conductance on hydrocarbon chain length

It has been shown that for both gramicidin and *o*-pyromellitylgramicidin the conductances of the unit channels in the present systems are not a function of

* That the adsorption of the *o*-pyromellitylgramicidin onto the lipid-water interfaces is strong (perhaps irreversible) seems likely if only because the transfer of the many lipophilic residues in an *o*-pyromellitylgramicidin molecule from an aqueous to a hydrocarbon environment could involve a free energy change of at least −15 to −20 kcal/mol.

the alkane retained in the membranes. It seems, therefore, that the large variations in high levels of membrane conductance observed for the different alkanes under conditions of constant polypeptide adsorption must reflect changes in the equilibrium which is believed to exist between conducting dimeric structures and non-conducting monomers. The basis of a possible explanation for the changes in this equilibrium was described in the Introduction and is illustrated in Fig. 5. A relationship between the equilibrium constants K and K_0 for pore formation in membranes in which a deformation is and is not required has been derived in the Appendix; i.e.

$$K = K_0 \exp\left[-\frac{\gamma \Delta A}{kT}\right] \tag{1}$$

where ΔA is the change in the membrane surface area produced by the deformation and γ is the surface tension of the membrane. Referring to Fig. 5, a rough estimate of ΔA is given by

$$\Delta A \approx 2\pi r(h - h_0) \tag{2}$$

h_0 being the length of the dimeric channel, h the thickness of the hydrocarbon region of the bilayer and r the radius of the idealized dimple. Combining Eqns. 1 and 2,

$$K \approx K_0 \exp\left[-\frac{2\pi r\gamma(h - h_0)}{kT}\right]. \tag{3}$$

The membrane conductance per unit area G may be expressed

$$G = \Lambda N_D \tag{4}$$

where Λ is the mean unit channel conductance and N_D is the number of dimeric conducting complexes per unit area. If

$$K = \frac{N_D}{N_M^2} \tag{5}$$

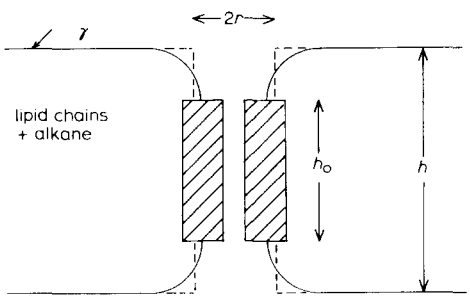

Fig. 5. A diagram of the theoretical model. The cross-hatched region represents an *o*-pyromellityl-gramicidin channel of length h_0. It is spanning a bilayer of hydrocarbon thickness h, whose surface tension is γ. The dashed lines indicate the simple parallel-sided dimple approximation used in the equations (see text). The radius of the idealized dimple is r.

where N_M is the number of monomeric polypeptides per unit area, then on combining Eqns. 3, 4 and 5 the membrane conductance may be written

$$\ln G \approx \ln G_o - \frac{2\pi r\gamma(h - h_o)}{kT} \tag{6}$$

G_o being the conductance when $h = h_o$. In obtaining Eqn. 6 it has been assumed that the equilibrium value of N_M is not a function of h. If the monomers on the membrane surface are in equilibrium with those in one or both of the bulk phases, then this should be so since dimerization could not significantly deplete the total number of available monomers. If there is no equilibrium with the bulk phases it is necessary that $N_M \gg N_D$, or that the polypeptide in the dimer form is always a small fraction of the total present in the membrane. Since the conductance (which is proportional to the number of dimers) declines rapidly with increasing alkane chain length, it is only for hexadecane that the number of monomers might be appreciably diminished. But even here, h is significantly larger than h_o and it is not obvious that there should be a serious diminution.

Figs. 2 and 4 show three series of experiments in which G has been measured while all of the parameters on the right-hand side of Eqn. 6, except for h and γ, have been held approximately constant. In Fig. 6 the data for those systems

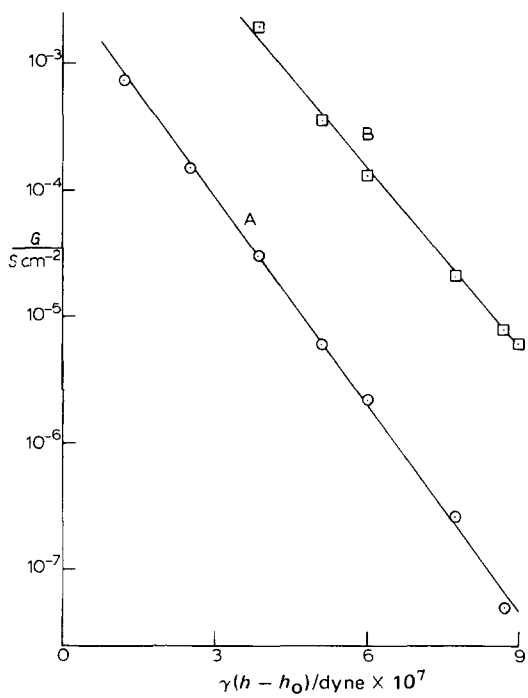

Fig. 6. Membrane conductance per unit area plotted against the product of membrane surface tension (γ) and the excess of membrane thickness over pore length ($h - h_o$). The approx. parallel straight lines are predicted by the theory expressed in Eqn. 6. The linear regression lines yield r = 8.1 Å (A) and r = 7.1 Å (B). A corresponds to the data of Fig. 4 whilst B is obtained from the data of Fig. 2.

TABLE I

THICKNESS AND INTERFACIAL TENSION FOR BLACK LIPID FILMS OF MONOOLEIN + ALKANE IN 0.1 M NaCl [19]

Alkane	Hydrocarbon region thickness (h)/Å	Interfacial tension (γ)/dyne cm^{-1}
n-Heptane	47.1	4.70
n-Octane	47.8	4.38
n-Decane	48.1	3.84
n-Dodecane	45.3	3.45
n-Tetradecane	40.7	3.04
n-Pentadecane	36.6	2.88
n-Hexadecane	32.7	2.51

in which h and γ are known (i.e. for 6 mM monoolein) have been plotted in the form of Eqn. 6. Values of h and γ have been extracted from [19] and are shown in Table I. (It should be remembered that since contact angles in the present systems are small, γ is given to a good approximation by the tension of the annular interfaces [14,22].) The value of h_0 has been taken as 28 Å [1,4]. As required by the equation, straight line plots are obtained, the slopes of which give r = 8.1 Å (A) and r = 7.1 Å (B). This is considered to be satisfactory since the outside radius of the pore can be estimated from molecular models to be approx. 10 Å. A further interesting feature of the plots in Fig. 6 is that even though the thickness, h, of the membranes becomes effectively constant for alkanes smaller than decane, the conductance continues to decrease, as expected from the fact that the membrane tension continues to rise (Table I).

Concluding remarks

The most striking aspect of the results is that the adsorption of the alkanes into the bilayers produces decreases in the membrane conductance which can be very large for the lower homologues. This quite clearly occurs through a loss of stability of the individual polypeptide channels. The explanation which has been proposed for the influence of the alkanes on the channel stability appears to account for the observations as well as can be expected, given that the model is crude and that there is necessarily some uncertainty over the radius of the dimple. The essential feature of the explanation is that in thick membranes having a significant tension, stresses are set up which prevent the pore from spanning the membrane. These stresses appear to operate in gramicidin systems by pulling the dimer apart in the middle, but they could equally well be effective in non-dimerizing systems simply by pulling one end of the pore away from an interface. For this reason, it is possible that the effects and mechanisms described could be of general importance in artificial and biological membranes, as suggested recently in papers on anaesthesia [23–26].

Appendix

As discussed in this and earlier papers, the dimerization of gramicidin in a lipid bilayer is thought to involve a deformation or dimpling of the membrane.

The energetics of this process may be described in a variety of ways which, however, divide broadly into (1) those based on the free energy of the membrane as a whole, and (2) those which consider only the free energy of the gramicidin [27,28]. In our present state of knowledge the two approaches involve essentially the same assumptions and, as the second is much the simpler to describe, it is this which is given below.

Under the conditions in which it is normally examined, the gramicidin in the bilayer membrane is very dilute, so much so that it does not produce a detectable change in tension ($\lesssim 0.1$ dyne cm^{-1}). In considering the reaction in which gramicidin monomers (M) form dimers (D), i.e.

$$2M \overset{K}{\rightleftharpoons} D \tag{1A}$$

it may be assumed, therefore, that both species form ideal two-dimensional monolayers at the surfaces of the lipid membrane. Expressions for the chemical potentials of the molecules under these conditions take the form [28]

$$\mu_M = \mu_M^{\circ} + kT \ln N_M \text{ and} \tag{2A}$$

$$\mu_D = \mu_D^{\circ} + kT \ln N_D \tag{3A}$$

where μ_M° and μ_D° are the standard chemical potentials in the membranes in question and N_M and N_D are numbers of molecules per unit area. From a statistical mechanical derivation of this type of equation [28] it can be seen that, in general, μ_M° and μ_D° include a term which describes the potential energy of the molecule relative to some reference state outside the membrane. For a membrane which is thicker than the length of the dimeric channel, the work done in transforming the dimer from the reference state into its conducting position includes the work needed to overcome the surface energy of deformation of the lipid. If the tension of the membrane is assumed to be unaffected by the dimerization, the energy required for the deformation may be written $\gamma \Delta A$, where γ is the surface tension of one side of the membrane and ΔA is the change in surface area per dimeric channel. (It should be noted that γ has no thermodynamic significance but may be thought of simply as $\sigma/2$, where σ is the tension of the membrane.) Obviously, no term such as $\gamma \Delta A$ is involved for the monomeric (non-conducting) species since it is assumed not to interact simultaneously with both sides of the membrane.

Eqn. 3A may therefore be rewritten

$$\mu_D = \mu_D^{\circ'} + \gamma \Delta A + kT \ln N_D \tag{4A}$$

and, using the equilibrium condition

$$2\mu_M = \mu_D \,, \tag{5A}$$

it is clear the equilibrium constant K of Eqn. 1 A is given by

$$K = \frac{N_D}{N_M^2} = \exp\left[-\frac{(\mu_D^{\circ'} - 2\mu_M^{\circ})}{kT}\right] \exp\left[-\frac{\gamma \Delta A}{kT}\right] \tag{6A}$$

or

$$K = K_0 \exp\left[-\frac{\gamma \Delta A}{kT}\right] \tag{7A}$$

where K_0 is the equilibrium constant for a membrane in which channel formation involves no deformation, i.e. $\Delta A = 0$.

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