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The Activation Enthalpies for Ion Conductance Systems in Lipid Bilayer Membranes

S. Ginsburg and D. Noble

University Laboratory of Physiology, South Parks Road, Oxford, England

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Summary. The activation enthalpies for various ion transport mechanisms through lipid bilayer membranes were measured using a feedback control system that allowed accurate control of temperature over the range 17 to 45 °C. In all cases (valinomycin, monactin, dibenzo-18-crown-6, benzo-15-crown-5, gramicidin A and alamethicin), the results give a simple straight line when presented as an Arrhenius plot, so that a single activation enthalpy characterizes the overall process in each case. The activation enthalpies for valinomycin and monactin are high and are similar to those expected for an electrostatic ("image") barrier in the hydrocarbon phase. The interpretation of the energies for the pore-formers is more complex. Alamethicin gives a very large activation energy that probably represents the energy for a co-operative process involved in the formation of each conducting unit. The results for gramicidin A are used, together with those of Hladky and Haydon (*Biochim. Biophys. Acta* **274**:294, 1972) to calculate limits on the values of the energy of formation of a channel and the activation energy for formation of a channel.

A large number of antibiotics and synthetic compounds are now known to enhance the transport of inorganic ions across lipid bilayer membranes. Among these, valinomycin, the actin antibiotics and gramicidin A have been studied extensively (Ciani, Eisenman & Szabo, 1969; Eisenman, Ciani & Szabo, 1969; Hladky & Haydon, 1970; Eisenman, Szabo, McLaughlin & Ciani, 1971; Stark & Benz, 1971; Haydon & Hladky, 1972). In general, the membrane conductance is found to be a function of several parameters: antibiotic concentration, inorganic ion concentration and species, bilayer composition, ionic strength and pH of the aqueous bathing solution, transmembrane voltage, time and temperature. Of these parameters, the concentration, voltage and time dependencies have proved most useful in elucidating the conductance mechanisms involved. The evidence suggests that some of the conductance-inducing compounds act as carriers while others act as pores. Thus, the antibiotics valinomycin and monactin act as

lipid-soluble carriers whereas gramicidin A acts as a pore. The evidence is discussed in a critical review by Haydon & Hladky (1972).

From an energetic point of view, these two mechanisms are very different. A lipid-soluble carrier provides a means for "dissolving" the ion in the hydrocarbon phase but its transport across the membrane still requires a charged particle to be taken across a low dielectric constant medium. The electrostatic ("image") energies (*see* Neumcke & Läuger, 1969; Haydon & Hladky, 1972) involved in crossing a medium of dielectric constant 2 about 50 Å thick are large (of the order of 30 to 50 kcal, depending on the size of the ion carrier complex). By contrast, a pore-former creates a hydrophilic channel of relatively high dielectric constant and much smaller energies for ion transfer may be expected.

It is of some importance, therefore, to study the activation enthalpies by investigating the temperature dependence of ion conductance. This is the aim of the experiments described in the present paper.

Mueller and Rudin (1967) published some data for gramicidin A, valinomycin and dinactin, but they do not give details and their data apply to membrane formed of mixed lipids of unknown composition. Since the present work was started, Hladky and Haydon (1972) have given conductance values for single gramicidin channels at two different temperatures (3 and 19 °C). These results confirm the general expectation discussed above, but the results are incomplete. In particular, without measuring conductance at a wide variety of temperatures one cannot exclude the possibility that the apparent activation enthalpy may be temperature dependent, as would be the case if the rate-limiting process were different over different temperature ranges. An Arrhenius plot might then show more than one slope.

Krasne, Eisenman and Szabo (1971) have already shown that in a special type of bilayer the membrane may be "frozen" and "melted" by changing the temperature. They found that the bilayer conductances in the presence of valinomycin and nonactin were greatly reduced on "freezing" the membrane, whereas the conductance in the presence of gramicidin A remained relatively unaffected. The simplest explanation is, of course, that the former act as carriers which are immobilized in a "frozen" layer, while the latter acts as a pore whose properties do not depend on the state of the hydrocarbon.

Materials and Methods

In our experiments, bilayers were formed from phospholipids or monoglycerides in tetradecane and the membranes were in the liquid state over the entire temperature range studied. Chromatographically pure egg lecithin was kindly supplied by A. Moore

(Radcliffe Infirmary, Oxford); phosphatidyl serine, glyceryl-monooleate and *n*-tetradecane, all chromatographically pure, were purchased from Sigma, London. The membrane-forming solutions were injected into a 1.6 mm aperture of a Teflon cell through a tunnel drilled in the cell wall. The cell was inserted into a larger Teflon vessel, thus obtaining a double compartment bath. Aqueous solutions contained 0.1 N NaCl (BDH analar grade), and the antibiotics were added to these solutions from concentrated stock solutions in methanol. Valinomycin was purchased from Calbiochem (Los Angeles), gramicidin A from Koch Light (England), dibenzo-18-crown-6 and benzo-15-crown-5 from Dupont de Nemours & Co. (N.Y.), monactin and alamethicin were gifts from Ciba-Geigy (Basel) and the UpJohn Company (Michigan), respectively.

A thermoelectric device (Cambion model 800-3450-01) was used to control the temperature. The device is a metal plate acting as a Peltier cell. In fact, two such units were employed to ensure maximal contact between the metal surfaces and the bottom of the Teflon bath. The bath, situated above the plates, was heated by driving current through the thermoelectric device in one direction. Cooling was obtained by driving current in the opposite direction. The pumping capacity was about 14 watts. The device functioned within the temperature range 5 to 65 °C. The temperature of the bath was kept constant within 0.3 °C by a feedback circuit using a thermistor situated inside the bath. The electric circuit (kindly designed and built for us by Mr. W. Laycock) and further details of the experimental apparatus are given elsewhere (Ginsburg, 1973; Ginsburg & Spindler, 1973).

A potentiometer was used to apply voltages up to 200 mV to the membrane. A digitimer (Devices) was employed to trigger pulses of set durations. Silver-silver chloride electrodes were used for applying the voltage and for collecting the current. The current was amplified by an operational amplifier which could measure current levels down to 10^{-11} amps. The circuit (designed by Mr. W. Laycock) is given elsewhere (Ginsburg, 1973).

Results

Membrane conductances were measured at a transmembrane potential of 20 mV. For potentials of this order of magnitude the current-voltage relations are approximately linear. At larger potentials the current-voltage relations become nonlinear and the voltage dependence of the conductance may then vary with temperature. We shall discuss this phenomenon in a subsequent paper (Ginsburg & Noble, *in preparation*).

For each of the antibiotics and polyethers used we found the conductance G was well described over most of the temperature range 17 to 45 °C by the simple relation

$$G = A \exp(-H_H/RT) \tag{1}$$

where A is a constant, H_H is the activation enthalpy for the overall transport process, R is the gas constant and T is the absolute temperature. This is illustrated by the Arrhenius plots shown in Fig. 1. These plots were obtained by averaging the results from between five and ten membranes in each case. Moreover, conductances were measured over the whole range of temperatures for each membrane so that difficulties arising from comparing

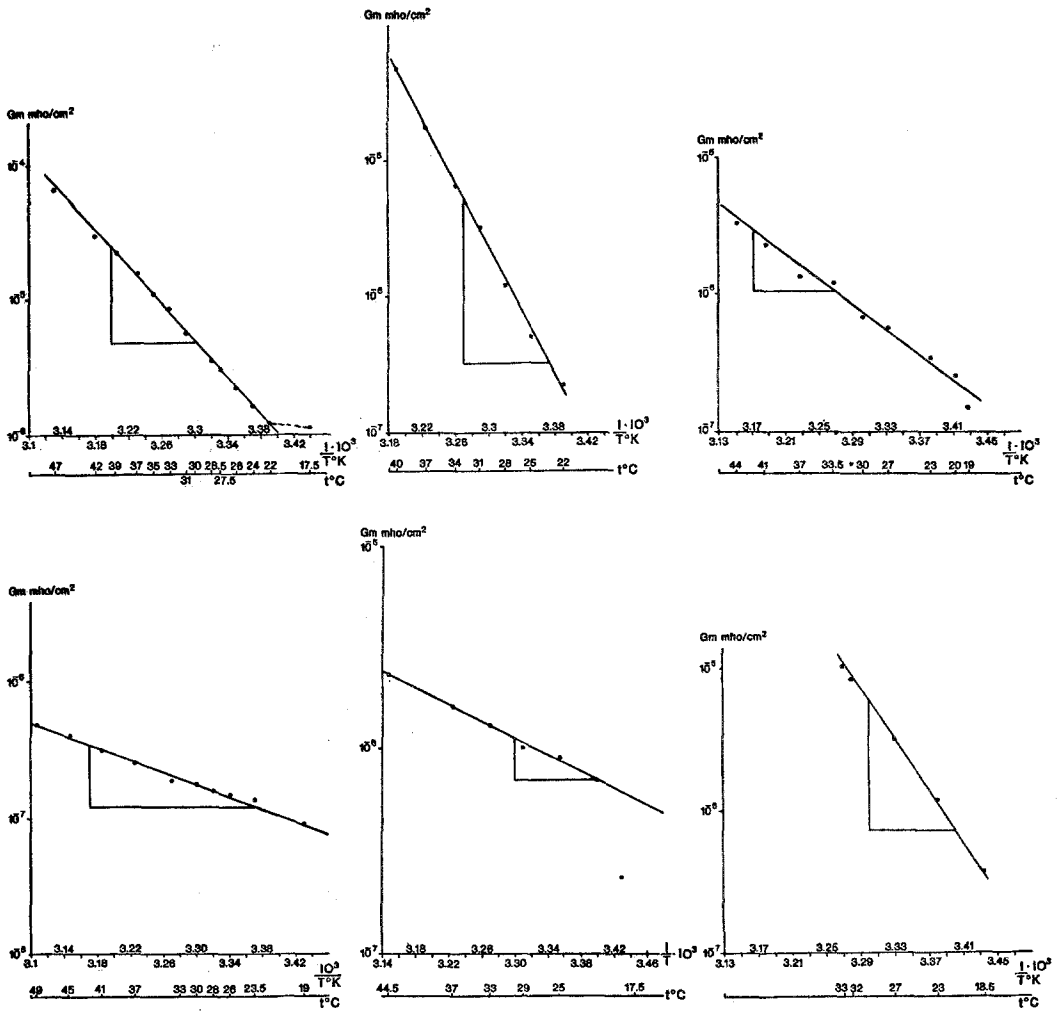

Fig. 1. Plots of log membrane conductance versus the reciprocal of absolute temperature. The top row of plots shows (from left to right) results for: monactin, valinomycin, and 18-crown-6. The bottom row of plots shows results for 15-crown-5, gramicidin A and alamethicin. These results show the averaged values for between five and ten membranes in each case. Examples of results for individual membranes are shown in Tables 2 and 3 and in Figs. 2 and 3

the conductances of different membranes at different temperatures were avoided. Finally, it is worth noting that no significant hysteresis was observed; i.e., the conductance measured in a given membrane at a given temperature was the same during increases and during decreases in temperature.

The points have been fitted in each case by a straight line whose slope is equal to $-H_H/R$. Table 1 summarizes the activation enthalpies calculated for each conducting system.

Antibiotic or polyether	Bilayer composition	Temperature range (°C)	Activation enthalpy (kcal/mole)
6×10^{-9} M monactin	PS/TD and lecithin/TD	17.5–47	32.5±0.5
2×10^{-8} M valinomycin	PS/TD and gmo/TD	22–40	55 ±1.0
10^{-4} M dibenzo-18-crown-6	gmo/TD PS/TD	19–44	21.5±0.5
10^{-6} benzo-15-crown-5	PS/TD lecithin/TD	19–50	10.3±0.3
Approx. 10^{-8} M gramicidin A	gmo/TD	17–45	9.3±0.6
Approx. 5×10^{-4} mg/ml alamethicin	gmo/TD	18–33	38.7±0.9

PS: phosphatidyl serine; gmo: glyceryl monooleate; TD: tetradecane.

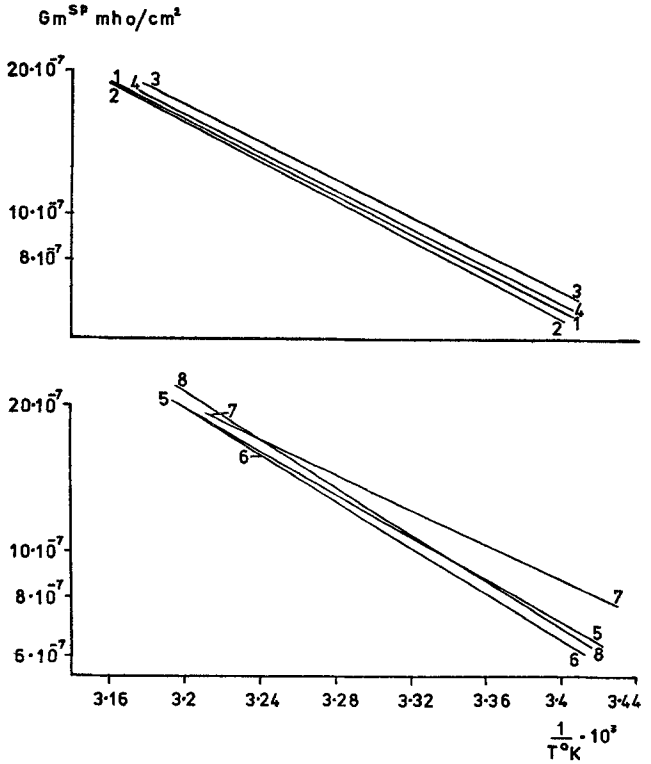

Fig. 2. Least-squares regression lines calculated for gramicidin results shown in Table 2. The number in each case identifies the membrane. Individual points are omitted since many of them overlap. The variance in each case was less than 0.1% when the values for 17.5 °C were omitted from the least-squares calculations. These values fell substantially (about 50%) below the regression lines shown (*cf.* Fig. 1, bottom row, center plot)

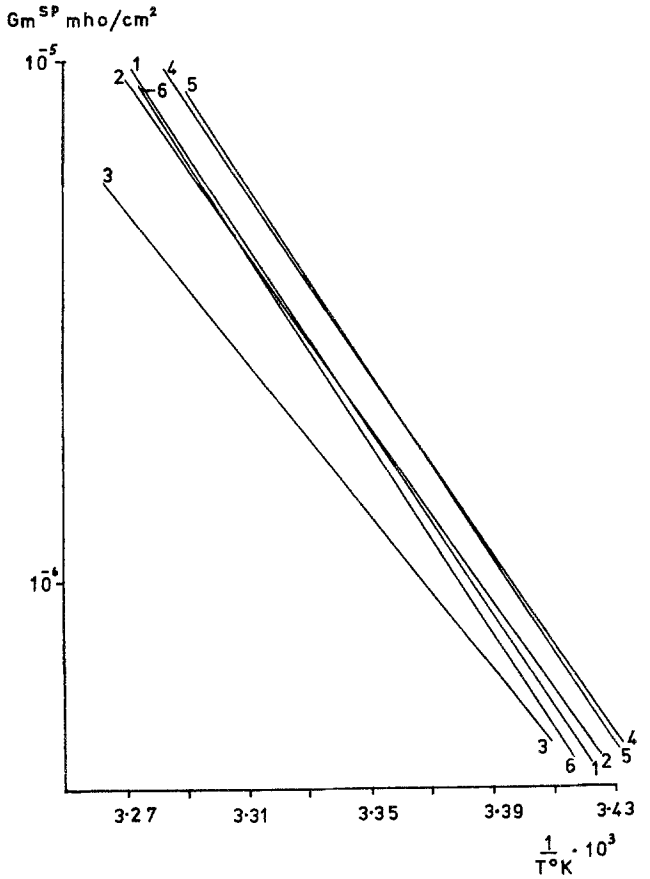

Fig. 3. Least-squares regression lines calculated for alamethicin results given in Table 3. The number in each case identifies the relevant membrane in the Table. In this case the conductance values for all temperatures were included in the least-squares calculations

Although the results shown in Fig. 1 were obtained by averaging the conductances measured at each temperature in several membranes, it is worth noting that very little variation was observed in the values for H_H obtained from different membranes, despite the fact that the absolute conductances in different membranes were different. This problem is particularly striking in the cases of gramicidin A and alamethicin which are sufficiently insoluble in both aqueous and membrane-forming solutions to make it difficult to control their concentrations. Figs. 2 and 3 and Tables 2 and 3 show the results for these two cases before averaging the measurements from different membranes. The diagrams show least-squares regression lines for each membrane. The figure on each line corresponds to the membrane number in the corresponding Table. In each case the variance

Table 2. Results for gramicidin (10^{-8} M) with NaCl (0.1 N) and 8 monoolein bilayers, at 20 mV

Membrane No.	Temperature (°C)						
	17.5	21	25	29	33	37	44.5
(1)	2.6	6.0	8.7	8.9	11.5	14.8	20.1
(2)	2.6	6.2	8.3	8.6	11.5	13.9	20.0
(3)	2.7	6.0	9.3	9.8	12.0	15.0	21.7
(4)	2.6	5.9	8.1	9.1	13.2	13.9	19.5
(5)	3.4	8.0	9.5	11.3	12.8	17.1	26.0
(6)	3.1	7.5	8.6	10.2	14.0	17.0	25.4
(7)	3.5	8.3	10.9	11.5	14.6	18.0	23.8
(8)	3.5	8.1	8.6	10.6	14.4	18.2	25.8

The numbers are values of G^{sp} in $(\text{mho/cm}^2) \times 10^7$.

Table 3. Results for alamethicin (5×10^{-4} mg/ml) with NaCl (0.1 N) and 6 monoolein bilayers, at 20 mV

Membrane No.	Temperature (°C)				
	18.5	23	27	32	33
(1)	3.5	11.0	30.1	82.0	105.0
(2)	3.4	11.3	33.6	79.5	100.0
(3)	2.0	9.3	16.5	45.0	57.5
(4)	5.2	14.1	41.0	110.5	122.5
(5)	5.1	13.5	39.1	105.5	128.5
(6)	3.0	9.8	28.2	75.5	98.5

The numbers are values of G^{sp} in $(\text{mho/cm}^2) \times 10^7$.

was less than 0.1 %. Moreover, there is relatively little variation in the slope for different membranes. Thus, the calculated values of H_B for each of the alamethicin membranes were 40.2, 38.4, 33.2, 39.25, 40.3 and 40.9 kcal.

For the gramicidin membranes, a similar result was obtained; i.e., very little variation was observed between different membranes apart from variations in absolute conductances. However, in each case, there was a tendency for the slope to increase at low temperatures; i.e., the conductances measured at the lowest temperature (17.5 °C) were significantly lower (approximately 50 % less) than expected. This effect is also seen in the averaged results plotted in Fig. 1. We think that this is a genuine effect since the errors involved in measuring G are much smaller than the difference between

the 17.5 °C results and the line fitting the results for higher temperatures. In calculating the value of H_H for gramicidin transport shown in Table 1 we therefore ignored the value of G at 17.5 °C since the results at all other temperatures (20 to 44.5 °C) were well-fitted by a single straight line.

At present we have no explanation for the unexpectedly low value of G at 17.5 °C. Further experiments using even lower temperatures would be required to establish whether a higher overall activation enthalpy occurs below 20 °C.

Discussion

The results given in Table 1 are only partially explained by the general expectation discussed in the Introduction that the activation enthalpy for a carrier mechanism should exceed that for a pore mechanism. The overall activation enthalpies for valinomycin transport (55 kcal/mole) and monactin transport (32.5 kcal/mole) are indeed much higher than that for the pore-former gramicidin A (9.3 kcal/mole), as expected. However, the activation enthalpy for alamethicin (41 kcal/mole) is also high, although this compound is thought to form pores.

It should be noted, however, that the expectation applies only to the activation enthalpy for the ion *transfer* process alone, whereas the experiments described here measure the activation enthalpies for the *overall* conductance process including the enthalpy for the *formation* of the conductance mechanism in addition to the activation energy for ion transfer through the mechanism once formed.

In the case of carriers like valinomycin[1] and monactin, this distinction may not be important since it is likely that the energy for the formation of the ion carrier complex is small compared to the electrostatic force opposing transfer of the complex across the membrane. The latter may be even larger than the values of H_H given in Table 1 since these values must include the enthalpy for entry of the carrier into the membrane in addition to the activation enthalpy for transfer across the hydrocarbon phase. The free energy of entry is negative since the lipid/water partition coefficients for carriers like monactin and valinomycin are larger than unity. Thus, Szabo,

1 There is considerable variation in the literature in the values obtained for valinomycin transport (compare our results with those of Stark, Benz, Pohl & Janko, 1972). This may possibly be attributed to the use of membranes of different composition. This problem is clearly important since the temperature coefficient of net conductance is actually negative in some cases (Benz, Stark, Janko & Läuger, 1973). The conclusions given in the present paper should not, therefore, be considered as applicable to all membranes regardless of composition.

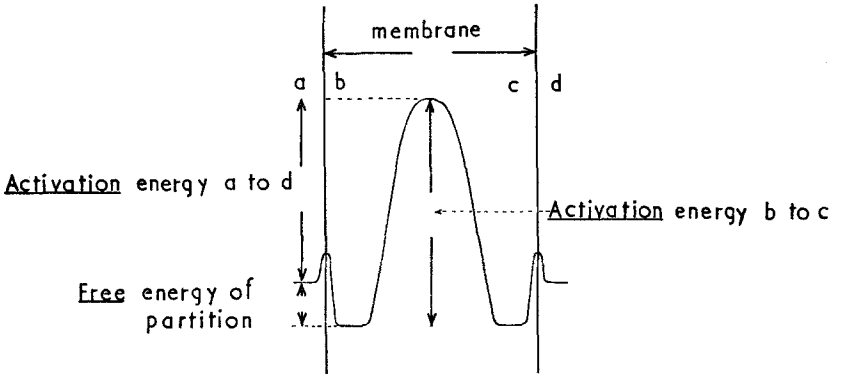

Fig. 4. Energy profile across the membrane when the electrostatic barrier is rate-limiting. The net activation energy (i.e., *a* to *d*) is formally the sum of two terms: the negative free energy of partition and the positive electrostatic activation energy barrier (i.e., *b* to *c*). If the free energy of partition is purely enthalpic, then the apparent (or experimental) activation enthalpy H_H is equal to the activation energy *a* to *d* in the diagram. It is then the sum of the negative free enthalpy of partition and the true electrostatic activation enthalpy *b* to *c*

Eisenman and Ciani (1969) obtained a partition coefficient of 5000 for uncomplexed nonactin. Similarly, Stark and Benz (1971) obtained a value of 25000 for valinomycin. A negative free energy for entry further increases the estimated value for the activation energy of transfer across the hydrocarbon phase (*b* to *c* in Fig. 4) since the net activation energy (*a* to *d*) will be an algebraic sum of the appropriate energy terms as shown in Fig. 4. This diagram illustrates the case when the electrostatic barrier is solely rate-limiting so that the entry process may be assumed to be in equilibrium and the relevant energy terms contributing to the total activation enthalpy are the free enthalpy of entry and the activation enthalpy of transfer across the hydrocarbon phase. The partition coefficients given above suggest that the negative energy of entry is several kilocalories.[2] It is difficult to be precise about this since the partition coefficients for complexed carriers may differ from those for free carriers (they are likely to be smaller, although this would be partly attributable to the electrostatic forces involved in moving a charged complex into the hydrocarbon phase), but it seems safe to assume that in the case illustrated in Fig. 4 the activation enthalpies for transfer across the hydrocarbon phase itself are somewhat larger than the experimen-

2 This is based on assuming the limiting case in which all the free energy of entry is enthalpic. If, however, a considerable fraction is entropic the partition coefficient will be less temperature dependent and the observed H_H will then more closely reflect the activation energy for hydrophobic transfer.

tal values obtained for the overall H_H. If, on the other hand, we assume that the entry process is rate-limiting then the situation becomes simpler and the observed activation enthalpy is that of entry and will be equal to the large energy values given in Table 1.

In the case of alamethicin, Mueller and Rudin (1968) and Gordon and Haydon (1972) have pointed out that the formation of a conducting system is a co-operative process involving six or more alamethicin molecules. This process may have a large activation energy. This point could be tested by measuring the activation enthalpies for unit conductances and it is hoped that this will be achieved in future experiments.

In the case of gramicidin A, the information presented here may be used, in conjunction with that for single gramicidin channels given by Hladky and Haydon (1972), to calculate limits on both the enthalpy of formation of a channel, H, and the activation energy of formation H_o. When small amounts of the antibiotic are added to the bathing solution, individual conducting units are observed. From the unit conductance values at two different temperatures, Hladky and Haydon (1972) estimated that the movement of sodium ions through the channel involves an activation enthalpy H_m of 4.9 kcal/mole. From a statistical analysis of channel lifetime at two temperatures, they estimated that the closing of a channel involved an activation enthalpy H_c of the order of 19 kcal/mole. When relatively large amounts of gramicidin A are added to the same bilayers, a high level conductance is observed. This conductance presumably originates from the summation of many individual conducting units. The activation enthalpy for the high level conductance, H_H, was calculated as 9.3 kcal/mole as seen in Table 1. H_H is a combination of two energy terms: (a) activation enthalpy for transfer itself (H_m) and (b) an energy term expressing the availability of conducting units. Thus, the high level conductance G is a temperature-dependent parameter, proportional to the unit conductance g as well as to the fraction x of open channels.

$$G(T)=g(T)\cdot x(T)\cdot n(T) \tag{2}$$

where n is the total number of gramicidin systems (open and closed) in the membrane expressed in terms of the total number of possible conducting systems. Multiplication and differentiation of the logarithmic form of this equation yields:

$$RT^2\frac{d\log G}{dT}=RT^2\frac{d\log g}{dT}+RT^2\frac{d\log nx}{dT}. \tag{3}$$

From Eq. (1) it is clear that the first term is H_H. Similarly, the second term equals H_m, the activation enthalpy for ion transfer through single channels.

Hence,

$$H_H - H_m = RT^2 \frac{d \log nx}{dT}. \tag{4}$$

Using the figures already given for H_H and H_m:

$$RT^2 \frac{d \log nx}{dT} = 4.4 \text{ kcal/mole}. \tag{5}$$

Eq. (5) expresses the temperature dependence of the number of open channels nx.

Further interpretation of the results is not easy since we must first know the temperature dependence either of n or of x to allow the other to be calculated. However, a possible upper limit on the temperature dependence of x may be obtained by assuming that n is nearly constant. This is equivalent to assuming *either* that the partition coefficient for gramicidin between water and membrane phases is not temperature dependent; i.e., that the free enthalpy for entry of gramicidin into the membrane is small *or* that the activation energy for entry or exit to or from the membrane is high enough to prevent significant variation in membrane concentration over the time course of a single experiment. The latter possibility may appear to be ruled out by the fact that only 5 to 10 min is required for the conductance to reach a steady level after forming a membrane when the gramicidin is freshly added to the aqueous phase from a methanol stock solution (Haydon & Hladky, 1972), as was done in our experiments. This argument is not conclusive, however, since the initial adsorption process may be relatively rapid but nevertheless irreversible. In either case, variations in n are assumed to be small. The consequences of relaxing this assumption will be discussed later.

We shall also assume that the formation of an open channel involves dimerization (i.e., that "closed channels" are nonconducting monomers and "open channels" are formed of two gramidicin molecules). The evidence for such a second-order reaction in the formation of gramicidin channels is strong (Bamberg & Läuger, 1973). We then have

$$2M \underset{k_c}{\overset{H, k_0}{\rightleftharpoons}} D$$

where M is a nonconducting monomer and D is a conducting dimer. H is the energy of formation[3] of the dimer and, provided that the entropy terms

3 This energy should be distinguished from the activation enthalpy of formation, H_o [*see* Eq. (12)].

are negligible, will therefore be related to the equilibrium concentrations of D and M by

$$D/M^2 = \exp(-H/RT). \tag{6}$$

The parameter x in Eq. (2) is the fraction of open channels expressed with respect to the total number of possible channels (i.e., to the total number of molecules divided by two). Hence,

$$x = D/(D + M/2) \tag{7}$$

and we obtain

$$D/M^2 = x/[2M(1-x)]. \tag{8}$$

Hence,

$$x/(1-x) = 2M \exp(-H/RT). \tag{9}$$

If H is significantly large (e.g., more than 3 kcal/mole), x will be small, $1 - x \to 1$, and

$$x = 2M \exp(-H/RT). \tag{10}$$

Moreover, when x (and D) are very small, M will be nearly constant so that differentiation of Eq. (10) gives

$$RT^2 \frac{d \log x}{dt} = H \tag{11}$$

and, from Eq. (5) with n constant,

$$H = 4.4 \text{ kcal/mole}.$$

Since

$$H_0 = H_c + H \tag{12}$$

where H_o is the activation enthalpy for channel formation, we also obtain

$$H_0 = 19 + 4.4 = 23.4 \text{ kcal/mole}.$$

Table 4 summarizes the values of the various energy terms obtained.

It must be emphasized, however, that the values for H and H_o are uncertain. Thus, if there is a significant enthalpy for entry of gramicidin into the membrane, n will increase with temperature and a smaller increase in x would be required to account for the total variation in nx given by Eq. (5). Hence, if the entry enthalpy is positive, the values calculated for H and H_o will be maximal values. The actual values must be smaller than those in Table 2.

Table 4. Energy values for gramicidin channels

Activation enthalpy for high level conductance, H_H (kcal/mole)	Activation enthalpy for unit conductance, H_m (kcal/mole)	Activation enthalpy for opening a channel, H_o (kcal/mole)	Activation enthalpy for closing a channel, H_c (kcal/mole)	Free Energy of formation of a channel, H (kcal/mole)
9.3	4.9	23.4	19	4.4

It is also conceivable that there may be a negative enthalpy for entry, as in the case of the carriers referred to earlier. Gramicidin would then prefer to lie in the membrane and n may decrease with temperature. All we may then conclude is that the algebraic sum of H and the enthalpy of entry may not exceed 4.4 kcal/mole. H itself could then exceed 4.4 kcal/mole by an amount determined by the enthalpy of entry into the membrane.

In addition to these difficulties in interpreting the gramicidin results, there is a major difficulty that applies to all studies involving measurements of overall activation enthalpies. This is that the energy values themselves may vary with temperature, or possibly also with voltage. In a subsequent paper (Ginsburg & Noble, *in preparation*) we shall present evidence that such variations do occur in the case of monactin transport. The interpretation of the overall activation enthalpy is then more complex and will be discussed in that paper.

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