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THE INFLUENCE OF STEROLS ON PENTACHLOROPHENOL-INDUCED CHARGE TRANSFER ACROSS LIPID BILAYERS STUDIED BY ALTERNATING CURRENT METHODS

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The frequency dependence of membrane admittance has been determined for a series of phosphatidylcholine /sterol/*n*-decane bilayers in the presence of an aqueous environment containing pentachlorophenol. Variations in the results among membranes can be related to differences in the kinetic parameters of a kinetic model of pentachlorophenol-induced charge transport by characterizing both measurements and model behavior in terms of a common equivalent circuit. The kinetic model assumes a three-layer structure for the membrane and immediate environment. Data from membranes formed with β-hydroxysterols having a flat ring structure and an intact side-chain (cholestanol, cholesterol, 7-dehydrocholesterol), after correction for sterol-induced membrane thinning, suggest that these sterols affect charge translocation by altering both interior fluidity and surface dipolar fields. The effects almost cancel for the case of cholesterol. These sterols also affect interfacial processes, either by inhibiting proton exchange between the aqueous and lipid environments, or by suppressing the adsorption of pentachlorophenol anions. Stigmasterol, coprostanol and epicholesterol cause only minor alterations in both translocation and interfacial processes. None of the sterols investigated has a significant influence on the capacitance of the interfacial region.

Introduction

The role of the sterol component in biological membranes has been the subject of a number of inquiries in recent years [1–3]. It has been suggested, for example, that a major function of cholesterol in plasma membranes is to moderate the fluidity of the hydrocarbon portion of the membrane against temperature changes and against variations in the fatty acid content of the component lipids [4,5]. Any comprehensive understanding of these matters must, however, take into account the distinctive features of the sterol composition found in membranes having particular biological function. For instance, the implications of the large differences in the mole ratios of sterol to total lipid content, which vary from 25–29% in mammalian erythrocyte to 1–5% in mitochondria to virtually zero in bacterial membranes [6], is not fully understood. In addition, the ubiquity of cholesterol as a membrane component in mammalian tissue as opposed to several other sterols with very similar structures is an important physiological consideration.

As with other questions relating to membrane functions, a great deal of insight can be gained by studying artificial model systems in which the composition and environmental conditions can be carefully controlled. For instance, Demel and coworkers [7,8] have in the course of their studies on lipid monolayers established that three aspects of sterol structure are essential for strong phospholipid-sterol interaction. Interestingly, these same structural criteria, viz., a planar sterol nucleus, a 3β-hydroxy group, and an intact side-chain, were also found to be correlated with strong inhibition

of permeability of phosphatidylcholine liposomes [9–11]. Steady-state electrical conductivity studies of lipid bilayers in the presence of lipophilic ions and charge carriers by Szabo and other workers have consistently shown a strong dependence on the relative richness in cholesterol of the membrane forming solution [12–14]. Also working with lipid bilayers, Benz and co-workers [15–17] have investigated the influence of a number of naturally occurring sterols on the transport kinetics of lipophilic ions and carrier complexes of both positive and negative charge, using charge pulse relaxation techniques. These relaxation studies have resulted in important additional information on the details of membrane transport in the presence of sterols, although some unanswered questions still remain. Compared with cholesterol, none of the sterols tested exhibited any strong influence on the transport properties. However, cholesterol had little influence on dipicrylamine transport in phosphatidylcholine membranes, whereas it had strong effects in monoolein membranes.

Although the relaxation studies have been able to provide much valuable information on the influence of sterols on kinetic parameters, complementary studies using other probes and dynamic techniques can be useful in clarifying the detailed features of sterol containing bilayers. Among these complementary methods, alternating current techniques may in certain cases offer the possibility of discerning differentially the influence of additives on the surface and interior portions of the membrane, as in the low-frequency studies of Coster and co-workers [18,19]. Our own experiments involve a measurement of the frequency dependence of membrane admittance in which the uncoupler pentachlorophenol is used as a probe substance. An analysis of the alternating current properties of a charge transport model for pentachlorophenol shows that under appropriate conditions the interfacial (surface) and translocation (interior) parameters of the model have distinguishible influences on the frequency dependence of membrane admittance.

We have chosen to investigate in this study a series of six sterols whose structures constitute systematic variations on the basic cholestanol configuration. The structures of three of these (cholestanol, cholesterol and 7-dehydrocholesterol) are identical except for the number of double bonds (zero, one and two, respectively) in the second ring of the sterol nucleus. They each feature a 3β-hydroxy group, an intact side-chain, and a planar ring structure. In addition we studied the following sterols: stigmasterol, which differs from cholesterol only in possessing a double bond and an extra methyl moiety in the side-chain; and coprostanol and epicholestanol, the *cis* isomer and the epimer, respectively, of choslestanol. In epicholestanol the -OH is α-oriented, while coprostanol is sharply bent along the common bond joining the first two rings of the sterol nucleus.

Materials and Methods

Materials

Bilayer membranes were formed by brushing an egg-phosphatidylcholine/sterol/*n*-decane solution across a hole (1.5 mm diameter) in a Teflon cup separating aqueous solutions of identical composition. The concentration of phospholipid in the membrane-forming solution was held constant at 5.5 μmol/ml. The electrolytic solution was prepared from 0.5 M KCl and a buffer comprising 0.2 M potassium phosphate/0.2 M potassium citrate/0.05 M boric acid. This solution was titrated with HCl to pH 5.4, the pH for which the steady-state membrane conductance is maximized [14]. Pentachlorophenol (Aldrich Chemical Co., Milwaukee, WI) was dissolved only in the electrolytic solution to a concentration of 25 μM. Temperatures were maintained at 22 ± 1°C.

Egg phosphatidylcholine was prepared and purified in this laboratory by a method described in Ref. 14. The sterols were obtained from several sources as follows: recrystallized cholesterol, a gift from Dr. D. McClure of the Chemistry Department; cholestanol from Aldrich Chemical Co.; 7-dehydrocholesterol and epicholestanol from Research Plus Steroid Laboratories, Inc., Denville, NJ; stigmasterol and coprostanol from Applied Science Laboratories, Inc., State College, PA. All these sterols were found to give a single spot when checked for purity using thin-layer chromatography.

Alternating current technique and equipment

A detailed discussion of the alternating current

method we have used in these experiments has been given in a previous publication [20]. We present here only a summary of essential points. The basic measurement is of conductance and capacitance of the membrane/measuring-cell system over a range of frequencies from 0.2 to 1000 kHz using an automatically balancing bridge (modified Hewlett Packard Type 4270A). Connection to the aqueous solutions is via platinized platinum electrodes. The applied voltage was in the large majority of cases less than 30 mV r.m.s. From the basic measurements, membrane conductances, G_m, and capacitances, C_m, are derived by correcting for the presence of a 'cell resistance' in series with the membrane. An essential point is that the cell resistance, because it is almost entirely determined by the conductance of the electrolytic solution close to the black area of the membrane, must be evaluated for each individual membrane by extrapolation to frequencies which are sufficiently high for the system to be considered as a pure membrane capacitance in series with a pure cell resistance. Finally, the experimental data are expressed in terms of loss tangent vs. frequency curves, where the loss tangent, tan δ, is the ratio $G_m/(\omega C_m)$, ω being the angular frequency. In every case, in addition to loss tangent curves obtained from membranes in the presence of pentacholorophenol ('treated membranes'), curves are obtained from membranes of identical composition for which no pentachlorophenol has been added to the aqueous solutions ('untreated membranes').

We have argued in the previous discussion of the alternating current method that subtracting at every frequency the loss tangent of untreated membranes from that of a corresponding treated membrane results in a loss tangent curve which is essentially due to pentacholrophenol-induced transport, the effects of dielectric losses in the membrane and background conductivity being essentially eliminated. The underlying assumption is that the loss mechanisms associated with the bare (untreated) membrane act in parallel with the transport mechanisms under study and are not appreciably altered by the presence of pentachlorophenol. In support of this assumption is the observation that the treated membrane capacitance does not differ significantly from that of the untreated membrane when measured at frequencies higher than those for which kinetic effects are important. A similar observation has been made in the case in which lipophilic ions are responsible for charge transfer (unpublished data). In any event, the correction is most important at the highest frequencies studied, which are weighted relatively lightly in the curve-fitting procedure described below.

In all our studies we have observed that the frequency dependence of the loss tangent which is obtained after subtraction of bare membrane losses mimics quite accurately the frequency dependence which would be generated by a 'three-element' capacitor-conductor circuit of the type shown in Fig. 1. Fitting of the experimental loss tangent data to curves based on the equivalent circuit enables us to find appropriate values of the circuit elements (G_1, C_1, G_2, C_2) to within a multiplicative constant which must be determined by an independent measurement of one of the parameters. This normalization is accomplished by setting C_2 equal to the measured specific capacitance at low frequencies, as a straightforward calculation shows that the equivalent capacitance of the circuit in Fig. 1 approaches that of the central element C_2 as $\omega \to 0$, as long as $G_1 \gg G_2$. The latter condition on conductances holds for all our studies. The curve fitting is based upon a least-squares procedure in which each experimental point is weighted according to its individual experimental error.

Theoretical considerations

Equivalent circuit and scheme of transport

In previous studies [20] a tentative interpretation of the 'three-element' circuit of Fig 1 was made in which the two identical outer elements consisting of conductor-capacitor pairs G_1 and C_1 represent the electrical properties of the polar surface regions of the bilayer, whereas G_2 and C_2 represent the hydrocarbon interior. Although such an interpretation obscures both the possibility that transport kinetics can contribute significant capacitive components to the membrane current as well as the effects of interior processes on surface conductances, it has proven to be useful in connection with a variety of studies of membrane modifiers

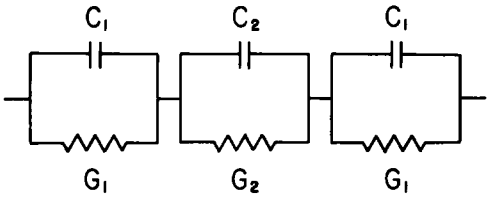

Fig. 1. Three-element equivalent circuit.

[18,19,21–23]. In the present study we choose to regard the equivalent circuit of Fig. 1 primarily as a convenient means for describing the experimental results. Indeed, investigations which one of us (J.H.) has carried out with P. Smejtek (unpublished data) have established that a number of alternative circuits are capable of generating curves of admittance vs. frequency which are indistinguishable from that of the three-element circuit. However, in this paper we show, in addition, that this type of admittance behavior is consistent with an accepted model of transport kinetics for pentachlorophenol-treated membranes. A detailed study of the model indicates that, in fact, for the experimental conditions of interest in this paper, G_1 characterizes the process of protonic transport across a polarizable interfacial region having a capacitance nearly equal to C_1, and G_2 describes the translocation of charged dimers across an interior region having a capacitance C_2.

The detailed scheme of pentachlorophenol-induced charge transport, which was deduced by Smejtek et al. [14] on the basis of steady-state measurements, is similar to schemes reported for several other second-order uncouplers [24,25]. The effective transfer of a proton (H^+) from one side of the membrane to the other, as it occurs in this scheme, is illustrated in Fig. 2. Neutral molecules (HA) and anions (A^-) of pentachlorophenol are adsorbed in the surface region on one side of the membrane where they combine to form charged dimers (HA_2^-), this being accompanied by the removal of protons to the aqueous solution; this is attended by a voltage driven translocation of dimers and a return flux of HA across the membrane. On the other side of the membrane, neutral molecules are again formed by the dissociation of dimers and the capture of protons from solution. During application of small alternating voltages

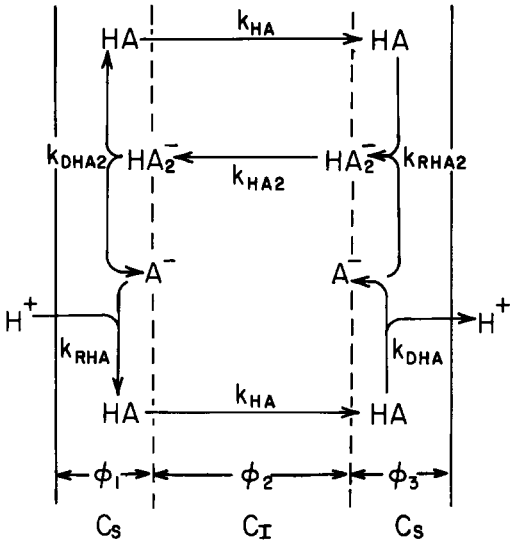

Fig. 2. Scheme of pentachlorophenol-induced charge transport. Interfacial charge transfer is assumed to occur across a surface region having effective capacitance C_s. Translocation of charged dimers takes place across an interior region with capacitance C_I.

only protons move to and fro between the aqueous phase and the surface region of the membrane, whereas other species involved in the transport process are slightly perturbed above and below their equilibrium values.

In explaining membrane alternating current properties on the basis of this model, we have found it necessary to include an additional refinement which takes into account the division of the applied voltage across the several regions of the membrane in which the kinetics may be thought to operate. We make this explicit in a form suggested by Ait'yan et al. [26] by assuming that there is a polarizable layer on either side of the membrane which separates the bulk aqueous solution from the adsorption-reaction plane for negative species (dashed line in Fig. 2); this surface region is characterized by a specific capacitance C_s. Between the charge containing planes is an interior region of specific capacitance C_I. Thus the rate constants for formation and dissociation of dimers and neutral molecules, the time average values of which (k_{RHA2}, k_{DHA2}, k_{RHA}, k_{DHA}) are indicated in Fig. 2, are dependent on that portion of the applied voltage which appears across the surface layer. The particular voltage dependence which is

assumed is that of the Eyring barrier type [27]. Similar considerations apply to the voltage dependence of the rate constant for dimer translocation (average value k_{HA2}). For the purposes of this paper it is sufficiently accurate to treat the model in the limit of very small applied voltages.

Details of the model calculations are given in the Appendix.

General performance of the alternating current model

In exploring the results of the model calculations outlined in the Appendix we have been concerned primarily with the possibility of variations in any of the kinetic parameters or combinations of parameters being associated with changes in the sterol content of the membranes, rather than with an attempt to determine precise values for the parameters. The strategy used to link the model-predicted alternating current behavior to the experimental findings is as follows. The measured results for all membranes are characterized in terms of the three-element circuit of Fig. 1 by the curve-fitting procedure previously discussed. In parallel with this, we seek sets of kinetic parameters which give rise to model-generated admittance data which are of similar form and likewise can be curve-fitted to yield equivalent circuit values. Thus, changes in experimental conditions can be related to changes in kinetic parameters through a series of equivalent circuits whose admittance behavior matches both the measurements and the model.

We began by finding sets of model parameters which correspond to a typical cholesterol-rich membrane at an intermediate pH (5.4) in the presence of $25 \cdot 10^{-6}$ M pentachlorophenol. The modeling was carried out in four different regimes of model parameters corresponding to the several extreme conditions which might characterize the surface reactions, as described below. A restriction was placed only on the value of the partition coefficient, β_{HA}, for adsorption of neutral molecules, β_{HA} being the ratio of the surface density of molecules in the membrane interface to the density in solution. β_{HA} was limited to the range between about 0.004 cm, which can be surmised on the basis of electrophoretic studies (Hsu, Jayaweera and Smejtek, unpublished data), and 0.008 cm, estimated by Pickar and Amos [20] on the basis of saturation effects in alternating current data. The pK for pentachlorophenol was assumed to be 4.8 [14]. The four kinetic parameter regimes are characterized in the following way:

(1) 'short-lived A^-' regime: $k_{RHA}(H^+) \gg k_{DHA2}$ and $k_{RHA2}[HA]_0 \gg k_{DHA}$, where (H^+) is the concentration of protons in the aqueous solution and $[HA]_0$ is the equilibrium surface density of adsorbed pentachlorophenol molecules on the membrane. For this situation, the production of the intermediate species A^- by the surface reactions proceeds very slowly compared to the rate at which the A^- is consumed to form either dimers or neutral molecules. In effect, the simultaneous generation of two neutral molecules HA accompanies the capture of a proton from solution, while a pair of HAs disappears upon release of a proton. This is essentially the scheme which was assumed for similicity in Ref. 20.

(2) 'Long-lived A^-' regime: $k_{DHA2} \gg k_{RHA}(H^+)$ and $k_{DHA} \gg k_{RHA2}[HA]_0$. The lifetime of the intermediate A^- is comparable with the time required for each surface reaction.

(3) $k_{RHA}(H^+) \gg k_{DHA2}$ and $k_{DHA} \gg k_{RHA2}[HA]_0$. For this regime of parameters there is a mixture of the surface rate limitations described in (1) and (2). The A^- intermediate produced by the dissociation of a dimer has a transitory existence, whereas the A^- produced by the dissociation of an HA molecule persists during the lifetime of the proton-releasing surface reaction.

(4) $k_{DHA2} \gg k_{RHA}(H^+)$ and $k_{RHA2}[HA]_0 \gg k_{DHA}$. The surface rate conditions are inverted compared to those in situation (3).

In all of the above cases, the model is capable of generating admittance versus frequency curves which resemble those found experimentally. We have not shown that any set of model parameters which provides a good correlation with a particular membrane measurement is unique; however, the conditions imposed on a good fit in each of the 'regimes' proved quite restrictive. In most cases some rate constants can be varied within limits without affecting the model calculations as long as certain ratios of constants are held fixed; these ambiguities have no effect on the conclusions of this paper. An example of the correspondence between model results and experiment for a cholesterol-rich membrane in the presence of pentachlorophenol is illustrated in Fig. 3. The curves

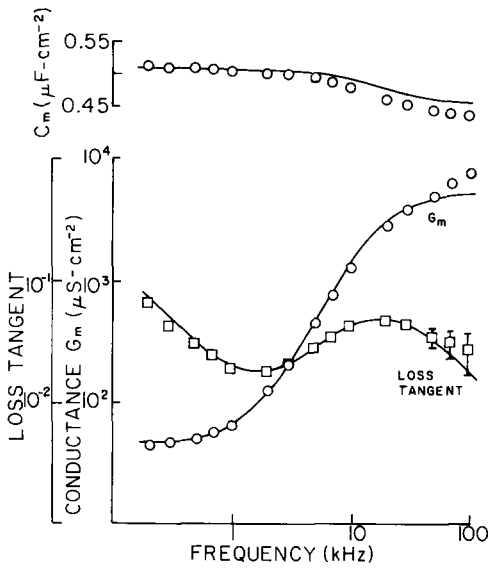

Fig. 3. Frequency dependence of membrane conductance, G_m, capacitance, C_m, and loss tangent $= G_m/(\omega C_m)$. Experimental points are those obtained from a membrane formed from a solution having a cholesterol mole ratio $Y = 0.7$ (sterol to total lipids) in the presence of 25 μM pentachlorophenol (pH 5.4). Shown here are net values obtained after substraction of bare membrane losses and correction for solution resistance. Error intervals are indicated only for loss tangent data. Solid curves are calculated on the basis of both the three-element circuit (Fig. 1) and a kinetic model (Appendix) using parameters giving the best weighted fit to the loss tangent data. Equivalent circuit parameters: $G_1 = 1.15$ S·cm^{-2}, $C_1 = 9.79$ μF·cm^{-2}, $G_2 = 44.8$ μS·cm^{-2}, $C_2 = 0.508$ μF·cm^{-2}. Kinetic model parameters: $\beta_{HA} = 4.0 \cdot 10^{-3}$ cm, $k_{RHA} = 2.38 \cdot 10^8$ M^{-1}·s^{-1}, $k_{DHA} = 1.50 \cdot 10^4$ s^{-1}, $k_{RHA2} = 2.50 \cdot 10^{13}$ M^{-1}·s^{-1}·cm^{-1}, $k_{DHA2} = 6.0 \cdot 10^6$ s^{-1}, $k_{HA} = 1.0 \cdot 10^6$ s^{-1}, $k_{HA2} = 445$ s^{-1}, $C_s = 9.70$ μF·cm^{-2}, $C_1 = 0.508$ μF·cm^{-2}.

generated by the model and the curves associated with the three-element circuit which gives the best weighted fit to the data are indistinguishable on the scale of the figure.

The kinetic models of a cholesterol-rich treated membrane appropriate to each of the four regimes above were further tested to determine their responses to changes in pH and pentachlorophenol concentration. Only for the 'long-lived A^-' model (parameter regime (2)) was the agreement with previous alternating current studies on pH and concentration dependence [20] reasonable. G_1 at lower pentachlorophenol concentrations was found to decrease approximately an order of magnitude for each two units of pH increase between pH 3.5 and 7.4, whereas C_1 was essentially unaffected by pH and concentration changes except for moderate increases upon lowering concentration at low pH. The major discrepancies with experiment appear to be related to the absence in the model of saturation effects, which the earlier studies indicate probably play a role at higher concentrations and lower pH values. It was also found that although the model utilizing parameter regime (3) gave good agreement with respect to G_1 changes, it predicted variations in C_1 very much larger than any which have been observed. The other two models were found to fail with respect to both the G_1 and C_1 dependence on pH and pentachlorophenol concentration. The set of parameters cited in the caption of Fig. 3 correspond to parameter regime 2.

Alternating current characteristics of the 'long-lived A^-' model

For realistic model conditions (pH 5.4, pentachlorophenol concentration $25 \cdot 10^{-6}$ M) and assuming parameter restrictions (2) (above), a systematic study of the effects of changing individual model parameters reveals that the behavior of the equivalent circuit conductances is described very closely by the following expressions:

$$G_1 \propto k_{DHA}\beta_{HA} \tag{1}$$

$$G_2 \propto \frac{k_{DHA}k_{RHA2}}{k_{RHA}k_{DHA2}}k_{HA2}(\beta_{HA})^2 \tag{2}$$

In terms of the equlibrium concentrations of charged species at the membrane surfaces these relationships can be written

$$G_1 \propto [A^-]_0 k_{RHA} \tag{3}$$

$$G_2 \propto [HA_2^-]_0 k_{HA2} \tag{4}$$

using Eqns. A11 and A14 and the proportionality $\beta_{HA} \propto [HA]_0$. For the 'long lived A^-' parameter regime, then, the 'outer' circuit conductance, G_1, is determined by the process which limits the rate of proton transport across the membrane interface, whereas the 'inner' circuit conductance, G_2, depends upon the translocation of dimers between interfaces. Circuit parameters are almost insensi-

tive to k_{HA}, except at such low values of this parameter that the model seriously fails to mimic the three-element circuit behavior.

As for equivalent circuit capacitances, C_1 has very nearly the same value as the model 'surface' capacitance C_s and, apart from low pH/low pentachlorophenol-concentration conditions, is practically independent of other model parameters. Likewise, the model-generated low frequency capacitance is in all cases almost identical to the 'interior' capacitance, C_1. This is analogous to the behavior of the three-element circuit for which calculations show that the equivalent capacitance at low frequencies is practically the same as C_2, as pointed out in our discussion of curve-fitting procedures. Thus for parameter regime (2) there are straightforward correspondences between the dielectric properties assumed in the model and the equivalent circuit capacitances, as well as between assumed charge transfer mechanisms and equivalent circuit conductances.

The relatively simple performance of the model just described is not a characteristic which necessarily applies when modeling is carried out in other parameter regimes. In general G_1, C_1, or both are found to depend in a complicated way on the kinetic parameters; in addition, the low frequency capacitance may differ appreciably from C_1. Indeed, the ability of the model to mimic the experimental findings best when applied in parameter regime (2) supports the interpretation of the three-element circuit made in previous studies, namely, that it represents, in the case of pentachlorophenol-doped membranes, three-layers having distinct charge-transfer and dielectric properties.

TABLE I

STEROL DEPENDENCE OF THE CENTRALLY LOCATED CAPACITANCE AND CONDUCTANCE IN THE 'THREE-ELEMENT' EQUIVALENT CIRCUIT REPRESENTING PENTACHLOROPHENOL-TREATED PHOSPHATIDYLCHOLINE MEMBRANES WITH CHOLESTANOL, CHOLESTEROL AND 7-DEHYDROCHOLESTEROL

Capacitance values C_2 which are assumed are those measured for untreated egg-lecithin/decane/cholesterol bilayers by Hanai et al. [29]. Values of hydrocarbon region thickness, d, are derived from C_2 assuming a dielectric coefficient, ϵ_H, of 2. Conductances G_2 are obtained from curve fitting to loss tangent versus frequency curves. g_2 values are conductances normalized to zero sterol content and corrected for electrostatic effects arising from membrane thinning. 25 μM pentachlorophenol, pH 5.4, 22°C.

Y (molar ratio)	$C_2(\mu F \cdot cm^{-2})$	d(nm)	$G_2(\mu S \cdot cm^{-2})$	g_2
0	0.38	4.9	15.6	1.0
Cholestanol				
0.2	0.38	4.9	18.2	1.2
0.4	0.39	4.7	17.1	0.9
0.5	0.42	4.5	23.1	1.1
0.7	0.51	3.7	23.9	0.5
0.75	0.55	3.4	28.6	0.4
Cholesterol				
0.2	0.38	4.9	20.0	1.3
0.3	0.39	4.8	21.0	1.2
0.5	0.42	4.5	29.2	1.3
0.7	0.51	3.7	41.6	0.8
0.79	0.58	3.2	85.3	0.8
7-Dehydrocholesterol				
0.2	0.38	4.9	13.4	0.9
0.3	0.39	4.7	15.0	0.8
0.4	0.39	4.7	19.3	1.1
0.6	0.46	4.1	66.7	2.1
0.69	0.50	3.7	260.0	5.1

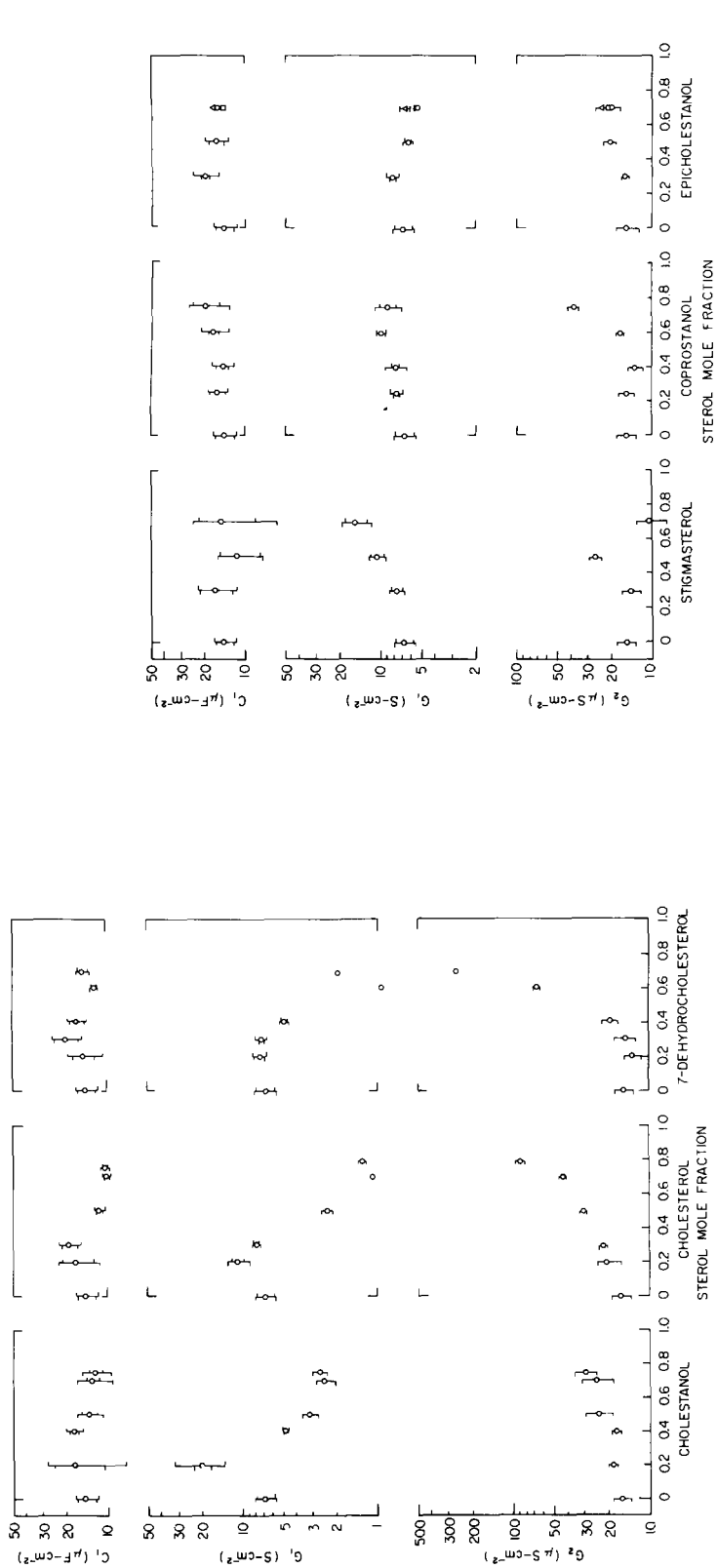

Fig. 4. Equivalent circuit parameters representing pentachlorophenol-treated membranes formed from solutions of egg phosphatidylcholine in *n*-decane containing various mole fractions (referred to total lipid) of the sterols cholestanol, cholesterol and 7-dehydrocholesterol. Error intervals indicated by flags pointing to the left are based on the standard error of the fit of the loss tangent data to the three-element circuit shown in Fig. 1. Error intervals indicated by flags pointing to the right represent the systematic errors arising from uncertainties in measuring solution resistance. Pentachlorophenol concentration 25 μM, pH 5.4.

Fig. 5. Equivalent circuit parameters representing pentachlorophenol-treated membranes formed from solutions of egg phosphatidylcholine in *n*-decane containing various mole fractions (referred to total lipid) of the sterols stigmasterol, coprostanol and epicholestanol. For interpretation of the error bars see caption to Fig. 4. Epicholestanol/phosphatidylcholine membranes contained some 7-dehydrocholesterol in the ratios (epicholestanol: 7-dehydrocholesterol) of 8 : 1 (squares), 4 : 1 (circles), and 2 : 1 (triangles). Pentachlorophenol concentration, 25 μM, pH 5.4.

Results

The results we have obtained for the sterol dependence of membrane electrical properties have for convenience been divided into two parts. The first of these, obtained with β-hydroxysterols having planar nuclei and intact side-chains (cholestanol, cholesterol and 7-dehydrocholesterol) is summarized in Fig. 4. The second group of results, involving stigmasterol, coprostanol and epicholestanol, is summarized in Fig. 5. Since we could not form sufficiently stable membranes using pure epicholestanol/phosphatidylcholine mixtures, a small amount of 7-dehydrocholesterol was incorporated in these membranes (ratios given in caption to Fig. 5). In view of the relatively small changes in the electrical properties which are observed when the ratio of epicholestanol to 7-dehydrocholesterol is varied from 8:1 to 2:1, the conclusions we have reached regarding the influence of epicholestanol is not much altered by the fact that another sterol was included.

The equivalent circuit conductances and capacitances shown in Figs. 4 and 5 are averages of values obtained, at each sterol-to-total-lipid mole fraction, Y, from measurements of at least three individual membranes. Two sets of error bars are associated with each value of 'outer' element conductance, G_1, and capacitance, C_1. One of these indicates the 'systematic' error arising in the reduction of the data from the uncertainty in cell resistance. This uncertainty has a negligible effect on the derived values of G_2. Another error bar drawn about each point in Figs. 4 and 5 indicates the 'random' error derived in the course of the curve fitting procedure; it represents the contribution of each circuit parameter to the standard error of the fit of the loss tangent information. In virtually all cases the scatter of the values from membrane to membrane is comparable to or smaller than this 'random' error. Apart from indicating an upper limit on the experimental scatter,

TABLE II

STEROL DEPENDENCE OF THE CENTRALLY LOCATED CAPACITANCE AND CONDUCTANCE IN THE 'THREE-ELEMENT' EQUIVALENT CIRCUIT REPRESENTING PENTACHLOROPHENOL-TREATED PHOSPHATIDYLCHOLINE MEMBRANES WITH STIGMASTEROL, COPROSTANOL AND EPICHOLESTANOL

Capacitance values C_2 which are assumed are those measured at low frequencies ($\leq$ 700 Hz) for untreated egg phosphatidylcholine/decane/sterol bilayers. Epicholestanol containing membranes were prepared from solutions containing some 7-dehydrocholesterol. (Molar ratios of epicholesterol to 7-dehydrocholesterol are in parentheses.) For conditions of measurement and a discussion of other data see heading of Table I.

Y (molar ratio)	C_2(μF·cm^{-2})	d(nm)	G_2(μS·cm^{-2})	g_2
0	0.38	4.9	15.6	1.0
Stigmasterol				
0.3	0.34	5.5	14.5	1.4
0.5	0.38	4.9	26.5	1.7
0.7	0.39	4.7	10.1	0.6
Coprostanol				
0.25	0.36	5.2	15.6	1.2
0.4	0.40	4.7	13.8	0.8
0.6	0.40	4.6	17.4	0.9
0.75	0.50	3.7	38.0	0.7
Epicholestanol				
0.3 (4:1)	0.38	4.9	15.9	1.0
0.5 (4:1)	0.38	4.9	20.2	1.3
0.7 (8:1)	0.35	5.3	20.8	1.8
0.7 (4:1)	0.35	5.3	19.5	1.7
0.7 (2:1)	0.35	5.3	23.2	2.0

this error is a measure of the validity of describing our results in terms of the three-element equivalent circuit.

Values of low-frequency specific capacitance, $C_m \simeq C_2$, which were used to normalize the other circuit parameters derived from the loss tangent curves are listed in Tables I and II. The uncertainties in the C_2 values (approx. $\pm 5\%$) were not included in the error estimates given for G_1, G_2 or C_1.

Discussion

Cholestanol, cholesterol, 7-dehydrocholesterol: variations in G_2, C_2

There is ample evidence [28] that a major effect of the inclusion of cholesterol in bilayer membranes formed from mixtures of lipid and decane is to decrease membrane thickness. This manifests itself by an increase in the specific capacitance as well as by an increase in the rate constant for translocation across the membrane of, for example, hydrophobic ions. In our own studies we observe that cholestanol as well as 7-dehydrocholesterol give rise, within experimental error, to the same sterol dependence of capacitance at low frequencies as does cholesterol. Thus, in order to facilitate comparisons among these sterols, the values of low-frequency capacitance we assume, as listed in Table I, are all taken from the curve on egg phosphatidylcholine/cholesterol/decane bilayers published by Hanai et al. [29]. In view of the kinetic model results on capacitance variations, as discussed above, we identify these values with both the circuit 'interior' capacitance, C_2, and the model capacitance, C_1. We assume that similar thickness changes, mainly in the hydrocarbon region of the membranes, are involved in the cases of all three sterols. Finally, in order to understand better the effect of these sterols on transport kinetics, we adjust our results on conductance to eliminate those aspects which are strictly thickness-related as follows.

Surface reactions are not expected to depend on thickness changes, whereas translocation rates should be affected. Our discussion of the kinetic model indicates that the experimental parameter which is in our situation sensitive to translocation rate constant changes is G_2; this circuit conductance is essentially proportional to k_{HA2} (Eqn. 4). The dependence on thickness of k_{HA2} can, in turn, be ascribed to electrostatic image forces which occur at the boundary between regions having different dielectric coefficients [30,31]. In particular, the height of the electrostatic energy barrier (in units of kT) across which a charged dimer HA_2^- must move changes by an amount

$$\Delta w(d) = h\left(\frac{1}{d_0} - \frac{1}{d}\right) \tag{5}$$

when the membrane thickness changes from a value d_0 to d. The parameter $h \simeq 18$ nm if the assumption is made that the dimer moves from a region bordering a highly polar phase across a hydrocarbon region with a dielectric coefficient $\varepsilon_H \simeq 2$ [28]. The thickness dependence of k_{HA2} is given by

$$k_{HA2}/k^0_{HA2} = e^{-\Delta w(d)} \tag{6}$$

where k^0_{HA2} is the rate constant for a sterol-free membrane of thickness d_0. Finally we express the dependence of G_2 on sterol mole fraction Y by

$$G_2(Y) = G_2(0)\, e^{-\Delta w(d)} g_2(Y) \tag{7}$$

where $g_2(Y)$ represents that portion of the normalized conductance which is influenced by sterol content in ways other than through thickness changes. In calculating $g_2(Y)$ from Eqns. 5 and 7, the values of d which are used are obtained from the specific capacitances listed in Table I by means of the parallel plate capacitance formula $C = \varepsilon_0 \varepsilon_H / d$. Values of $g_2(Y)$ are also listed in Table I.

Following other authors [13,16,32], we can suppose that the influence of sterols on charge translocation has, apart from thickness effects, two origins: (a) through chain-chain interaction with phospholipid, sterol induces a more viscous gel-like conformational state in the hydrocarbon layer; (b) sterol in the bilayer, because of the contribution of its own dipole field, raises the electrostatic potential in the interior of the bilayer, thereby lowering the barrier for translocation of negative species and/or augmenting the equilibrium concentration of these ions at the surface. The chief consequence of (a) is to lower the conductance of the membrane interior, whereas (b) results in an enhancement of conductance.

The data on $g_2(Y)$ given in Table I support an interpretation in which both fluidity and electrostatic effects influence interior conductance. The former effects appear to dominate in cholestanol-containing membranes, while the latter are most important when 7-dehydrocholesterol is the membrane sterol. There is more or less a cancellation of these effects in the case of cholesterol. This is consistent with previous studies which show little influence of cholesterol content on the translocation rates of various hydrophobic ions in constant thickness dioleoylphosphatidylcholine membranes [16,28].

The above discussion assumes, in accordance with the results obtained from our numerical modelling of pentacholorophenol-induced transport, that G_2 depends primarily on the rate constant, k_{HA2}, rather than on k_{HA}. If, however, for the sake of argument, we suppose instead that gradient-driven diffusion of neutral molecules is the rate-limiting translocation process, we are led to some inconsistencies. For this situation the thickness dependence can be taken into account by replacing the factor $\exp[-\Delta w(d)]$ in Eqn. 7 with a correction factor lying between 1 and d_0/d. The lower value of the factor pertains to the situation in which the free energy of the neutral pentachlorophenol molecule in its adsorption site at one interface differs greatly from its free energy in the interior, so that the localized desorption step into the interior is the controlling process; the upper value pertains to the case in which the molecule readily enters the interior region and the diffusion coefficient is assumed to be uniform throughout this region. However, calculations of $g_2(Y)$, modified on the basis of either of these extreme assumptions, lead to values which rise with increasing sterol mole fraction in all cases. This is contradictory to the evidence that sterol inhibits diffusion, thereby lowering k_{HA} and, with it, G_2. Indeed, all our modeling studies show that lowering k_{HA} is associated only with decreases, however small, in values of G_2.

Cholestanol, cholesterol, 7-dehydrocholesterol: variations in G_l, C_l

In contrast to the results on G_2, which, especially after correction for membrane-thinning effects, show only moderate changes below a sterol mole fraction of about 0.6, the 'outer' element circuit conductance G_1 exhibits, in all cases of the three β-hydroxysterols discussed here, approximately order of magnitude decreases between $Y = 0.2$ and $Y = 0.7$. These changes are accompanied by only small variations in the associated circuit capacitance, C_1.

Insofar as one can be guided by our model calculations, these results suggest the possibility that the inclusion of sterol in the membrane can inhibit proton exchange between membrane and environment. According to Eqns. 1 and 2, simultaneous decreases in the rate constants governing interfacial proton transfer (k_{DHA} and k_{RHA}), with the ratio k_{DHA}/k_{RHA} held constant, leads to decreases in G_1 accompanied by negligible changes in G_2 and C_1. Another explanation for the prominent drop in G_1 involves simultaneous decreases of k_{DHA} and k_{DHA2}; this is equivalent to a decline in surface anion concentration $[A^-]_o$ with increasing sterol content. In any event, it appears that each of the three β-hydroxysterols being considered here has a strong influence on the surface properties of the membrane.

As for the physical basis for these effects, surface dipolar changes induced by sterol does not offer an entirely satisfactory explanation for changes in interfacial transport. Studies on lipophilic ion transport [13,17] indicate that cholesterol alters dipolar fields in a direction which could inhibit positive-charge transport from aqueous solution across the membrane interface. In fact, Benz and Cros [16] observed order of magnitude decreases of the rate constant for complex formation k_R in the valinomycin-mediated Rb^+ transport system due to the inclusion of cholesterol in monoolein membranes; the dissociation rate constant, k_D, was unaffected. The changes in k_R were accounted for in terms of dipolar field variations induced by sterol which inhibited the transport of Rb^+ from the aqueous medium into the surface region of the membrane. By supposing that the complex is on the hydrocarbon side of the dipolar layer, the insensitivity of k_D to membrane composition was explained. Our data, on the other hand, suggest that it is the dissociation rate for the surface charge transfer reaction which is inhibited by sterol; moreover, the data require that other rates must be simultaneously affected. In particular, it is

difficult to imagine how dipolar fields could change k_{DHA} and k_{RHA} in the same, rather than in opposite, directions.

We have also considered whether structural modifications which alter the dielectric coefficient or thickness of the dipolar region of the membrane might play a role in inhibiting proton exchange. For instance, recent studies by Simon et al. [33] indicate that cholesterol decreases the depth of water penetration into bacterial phosphatidylethanolamine bilayers. According to our model calculations, any accompanying changes in the effective surface region capacitance, C_S, should be reflected in changes in the equivalent circuit 'outer' capacitance, C_1. Unfortunately, our experiment is unable to discriminate any such modifications. It is possible that the influence of sterol on the 'outer' conductance, G_1, which we observe, can be explained on the basis of some structural changes which strongly affect the adsorption of A^-. Electrophoretic studies would be helpful in resolving this. We note finally that the increases in G_1 which take place at very low sterol concentration may be related to the stearic rearrangements below mole fraction 0.33 suggested by the studies of Lentz et al. [34] on egg phosphatidylcholine cholesterol dispersions.

Stigmasteral, coprostanol, epicholesterol

We have incorporated these three sterols into our membranes in order to explore the effects on electrical properties of variations in the basic cholestanol configuration other than the degree of saturation in the sterol nucleus. Table II summarizes our results for C_2, G_2 and for the thickness adjusted 'inner' element circuit conductance, g_2. These data show relatively little systematic dependence on the concentration in the membrane forming solution of the three sterols being considered in this section.

Our results on g_2 are consistent with those obtained from relaxation studies using lipophilic probes [16,17]. The relaxation measurements indicate that of the sterols previously tested ergosterol and epicholesterol have little influence on the rate of ion translocation in phosphatidylcholine membranes, and that cholesterol, stigmasterol and dihydrocholesterol have moderate effects. It has been suggested that certain sterols are excluded to some extent from the black region of the membrane during thinning. This might also explain the small influence of certain sterols on capacitance C_2. Direct measurements relating the proportion of sterol in a bilayer to the concentration in the forming solution has thus far been made only in the case of cholesterol [34]. However, studies of Demel and co-workers [9] show that the degree to which most of the sterols studied in our work are incorporated into egg phosphatidylcholine liposomes varies, in fact, only over a range of about $\pm 20\%$ (molar ratio). Besides this, it is clear from our measurements of the 'outer' element conductance, G_1, that, at least in the case of stigmasterol, a moderate amount of interaction takes place with the phospholipid.

Conclusions

In these studies we have sought to ascertain the significance of several structural features of the sterol molecule for the electrical properties of pentachlorophenol-treated phosphatidylcholine/sterol bilayers. The experimental technique which was used involves the measurement of membrane admittance over a wide range of frequencies, the results of which can be described with good accuracy in terms of an equivalent circuit consisting of three resistor-capacitor pairs. The admittance spectrum of such a circuit, when compared with that predicted from an analysis of a kinetic scheme of pentachlorophenol transport, shows that the 'outer' circuit conductance is, under our experimental conditions, sensitive to interfacial processes. Sterol-induced changes in the 'inner' element conductance probably reflect modifications of interior translocation processes.

Of the six sterols investigated, membranes made with the three that share the criteria of Demel et al. [8,9] for strong sterol-phospholipid interaction in monolayers and for effective inhibition of permeability in egg phosphatidylcholine liposomes, exhibit a systematic pattern of electrical behavior which is, in fact, consistent with these properties. Until now it has not been known whether these structural characteristics were also significant for electrical behavior in phospholipid bilayers, as, of these three, only cholesterol had been so examined, and it showed only minor effects. Our

studies indicate that, indeed, cholesterol has only a small influence on charge translocation, whereas cholestanol and 7-dehydrocholesterol are more effective in this respect. However, all three cause large modifications in the interfacial charge transfer processes.

The differences among cholestanol, cholesterol and 7-dehydrocholesterol, so far as their influences on charge translocation are concerned, may be due to variations among them in their contributions to the dipolar fields near the membrane surface. This explanation also requires that their effects on the mechanical properties of the membrane interior are comparable, an assumption which is supported by liposome diffusion studies [9]. The number of double bonds in the sterol nucleus is possibly correlated with the orientation of or the charge separation associated with the hydroxyl group, which is responsible for the sterol dipole moment. Another explanation is that these sterols are located in the membrane at different depths, in the following order: cholestanol (least deep), cholesterol, 7-dehydrocholesterol. This latter conjecture is supported by our findings regarding the influences on interfacial reactions wherein cholestanol causes the most and 7-dehydrocholesterol the least radical modifications. However, the physical basis for these surface modifications is not entirely clear.

As in other studies, we show that sterols having either an α-oriented hydroxy group, a puckered ring structure or an interrupted side-chain have weaker influences in phospholipid layers. However, we cannot conclude, as in previous electrical studies, that stigmasterol and cholesterol are comparable in their effects on phosphatidylcholine bilayers. At least with respect to interfacial reactions, cholesterol produces large changes, whereas stigmasterol yields significant but much smaller modifications.

In the development of any comprehensive explanation of the role of sterol in biological function, the findings reviewed above ultimately have to be taken into account. For instance, our results suggest that in biological problems where modification of the membrane surface is important, such as in understanding the basis of certain drug action, the role of the sterol component may be of importance. Our use of pentachlorophenol in these alternating current studies grows out of an interest in the mechanisms of uncoupling action, but as a probe sensitive to both interior and interfacial properties it provides additional information on the role of sterol structure in determining membrane behavior. The interpretation of our experiments can be further enhanced by certain recent improvements in the experimental technique. Among these, the discrimination of effective interfacial capacitance, which is a membrane parameter unique to alternating current methods, is facilitated by increasing the rate at which admittance measurements are made, thereby reducing drift errors at high frequencies.

Appendix

The alternating current properties implied by the dimeric transport model diagrammed in Fig. 2 can be calculated using a formalism similar in some respects to that used by Ait'yan et al. [26] in their analysis of monomeric carrier schemes. The impressed voltage, ϕ, can be expressed as

$$\phi = \phi_1 + \phi_2 + \phi_3 \tag{A1}$$

where ϕ_1 is the component of the voltage across the surface layer bounded by the aqueous solution and the adsorption-reaction plane on the left-hand side of the membrane (specific capacitance C_s), ϕ_2 is the component across the region interior to the two adsorption-reaction planes (capacitance C_I), and ϕ_3 is the component across the right-hand surface region (capacitance C_s). These component voltages are given by

$$\phi_1 = \frac{1}{C'}\left\{ C_I\phi + F\left(1+\frac{C_I}{C_s}\right)\left(\Delta[A^-]' + \Delta[HA_2^-]'\right) + F\frac{C_I}{C_s}\left(\Delta[A^-]'' + \Delta[HA_2^-]''\right)\right\} \tag{A2}$$

$$\phi_2 = \frac{1}{C'}\{C_s\phi - F(\Delta[A^-]' - \Delta[A^-]'' + \Delta[HA_2^-]' - \Delta[HA_2^-]'')\} \tag{A3}$$

$$\phi_3 = \frac{1}{C'}\left\{ C_I\phi - F\frac{C_I}{C_s}\left(\Delta[A^-]' + \Delta[HA_2^-]'\right) - F\left(1+\frac{C_I}{C_s}\right)\left(\Delta[A^-]'' + \Delta[HA_2^-]''\right)\right\} \tag{A4}$$

where F is the Faraday constant, the parameter $C' = C_s + 2C_1$, $\Delta[A^-]'$ and $\Delta[A^-]''$ are the surface concentrations in excess of the equilibrium value of pentachlorophenol anions in the left- and right-hand adsorption-reaction planes respectively, and $\Delta[HA_2^-]'$ and $\Delta[HA_2^-]''$ refer to the analogous concentrations of charged dimers.

The surface concentrations of the reacting species participating in the transport process vary in time according to the following differential equations (Eqns. A5–A10) in which the voltage dependence of the rate constants are given in explicit form:

$$\frac{d[HA_2^-]'}{dt} = -[HA_2^-]'k_{HA2}\exp\left(-\frac{F\phi_2}{2RT}\right)+[HA_2^-]''k_{HA2} \times \exp\left(\frac{F\phi_2}{2RT}\right)+k_{RHA2}[HA]'[A^-]' - k_{DHA2}[HA_2^-]' \quad (A5)$$

$$\frac{d[HA_2^-]''}{dt} = [HA_2^-]'k_{HA2}\exp\left(-\frac{F\phi_2}{2RT}\right)-[HA_2^-]''k_{HA2} \times \exp\left(\frac{F\phi_2}{2RT}\right)-k_{DHA2}[HA_2^-]'' + k_{RHA2}[HA]''[A^-]'' \quad (A6)$$

$$\frac{d[A^-]'}{dt} = k_{DHA2}[HA_2^-]'-k_{RHA2}[HA]'[A^-]' + k_{DHA}[HA]'\exp\left(-\frac{F\phi_1}{2RT}\right) - k_{RHA}(H^+)[A^-]'\exp\left(\frac{F\phi_1}{2RT}\right) \quad (A7)$$

$$\frac{d[A^-]''}{dt} = k_{DHA2}[HA_2^-]''-k_{RHA2}[HA]''[A^-]'' + k_{DHA}[HA]''\exp\left(\frac{F\phi_3}{2RT}\right)-k_{RHA}(H^+)[A^-]'' \times \exp\left(-\frac{F\phi_3}{2RT}\right) \quad (A8)$$

$$\frac{d[HA]'}{dt} = k_{DHA2}[HA_2^-]'-k_{RHA2}[HA]'[A^-]' + k_{RHA}(H^+)[A^-]'\exp\left(\frac{F\phi_1}{2RT}\right)-k_{DHA}[HA]' \times \exp\left(-\frac{F\phi_1}{2RT}\right)-k_{HA}([HA]'-[HA]'') \quad (A9)$$

$$\frac{d[HA]''}{dt} = k_{DHA2}[HA_2^-]''-k_{RHA2}[HA]''[A^-]'' - k_{DHA}[HA]''\exp\left(\frac{F\phi_3}{2RT}\right)+k_{RHA}(H^+)[A^-]'' \times \exp\left(-\frac{F\phi_3}{2RT}\right)+k_{HA}([HA]'-[HA]'') \quad (A10)$$

In the above expressions the primes and double-primes refer to the left and right surfaces, respectively, and the surface concentrations of permeable species are the total concentrations, i.e., $[HA_2^-]' = [HA_2^-]_0 + \Delta[HA_2^-]'$, $[HA]' = [HA]_0 + \Delta[HA]'$, etc., where the zero subscripts denote the equilibrium concentrations. (H^+) is the concentration of protons in the bulk aqueous solution.

In the absence of an applied voltage, Eqns. A5–A10 reduce to the equilibrium expressions

$$k_{RHA2}[HA]_0[A^-]_0 = k_{DHA2}[HA_2^-]_0 \quad (A11)$$

$$k_{DHA2}[HA_2^-]_0 + k_{DHA}[HA]_0 = k_{RHA2}[HA]_0[A^-]_0 + k_{RHA}(H^+)[A^-]_0 \quad (A12)$$

$$k_{DHA2}[HA_2^-]_0 + k_{RHA}(H^+)[A^-]_0 = k_{RHA2}[HA]_0[A^-]_0 + k_{DHA}[HA]_0 \quad (A13)$$

Equations (A12) and (A13) are equivalent to Eqn. (A11) and the expression

$$k_{RHA}(H^+)[A^-]_0 = k_{DHA}[HA]_0 \quad (A14)$$

If a small voltage which is harmonic in form

$$\phi = \phi_0\exp(j\omega t) \quad (A15)$$

is applied, the perturbations from equilibrium of the surface concentrations will, in the steady state, vary sinusoidally in time with an angular frequency, ω. We may then everywhere replace $\Delta[HA_2^-]'$ by $\Delta[HA_2^-]'\exp(j\omega t)$, $\Delta[A^-]'$ by $\Delta[A^-]'\exp(j\omega t)$, etc., where the amplitudes $\Delta[HA_2^-]'$, $\Delta[A^-]'$, etc., are now regarded as time-independent complex quantities. We restrict our consideration of the problem to the case of small voltage amplitudes. We can then use the approximations

$$\exp\left(\frac{\pm F\phi_1}{2RT}\right) = 1 \pm \frac{F\phi_1}{2RT}$$

$$\exp\left(\frac{\pm F\phi_2}{2RT}\right) = 1 \pm \frac{F\phi_2}{2RT}$$

$$\exp\left(\frac{\pm F\phi_3}{2RT}\right) = 1 \pm \frac{F\phi_3}{2RT} \qquad \text{(A16)}$$

Furthermore, in the limit of very small voltages, after substituting Eqns. A2, A3, A4, A15 and A16 into Eqns. A5–A10 we can neglect higher order terms such as those involving $(\Delta[HA_2^-]')^2$, $\Delta[HA_2^-]'\Delta[HA]'$, $\Delta[HA_2^-]'\phi_0$, etc. Time-independent terms are eliminated by virtue of the equilibrium expressions Eqns. A11 and A14. Finally, because the membrane current depends only on the difference between concentrations of similar species at opposing interfaces, the total number of expressions in the system can be reduced by subtracting successive pairs of equations.

The resulting set of three inhomogeneous linear equations in $\Delta[HA_2^-]' - \Delta[HA_2^-]''$, $\Delta[A^-]' - \Delta[A^-]''$, and $\Delta[HA]' - \Delta[HA]''$ can be solved to permit computation of the membrane admittance

$$Y_m \equiv G_m + j\omega C_m = I/\phi \qquad \text{(A17)}$$

where the membrane current, I, is given by

$$I = F^2[HA_2^-]_0 k_{HA2}\frac{\phi_2}{RT} - Fk_{HA2}(\Delta[HA_2^-]' - \Delta[HA_2^-]'')$$
$$\times \exp(j\omega t) + j\omega C_1\phi_2 \qquad \text{(A18)}$$

We make no attempt in this work to write out the final expressions for membrane admittance in closed form; instead, we have used the above results to obtain numerical values of membrane conductance G_m and capacitance C_m as a function of frequency assuming various model parameters.

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