Biochemical Pharmacology, Vol. 23, pp. 2939–2950. Pergamon Press, 1974. Printed in Great Britain.

EFFECTS OF AMILORIDE AND DIBUCAINE ON A MODEL PHOSPHOLIPID MEMBRANE—STRUCTURE–ACTIVITY RELATIONSHIPS

MICHAEL A. SINGER

Department of Medicine, Queen's University, Kingston, Ontario, Canada

(*Received* 7 *January* 1974; *accepted* 28 *February* 1974)

Abstract—The local anesthetic dibucaine, and the diuretic agent, amiloride, both decrease the negative surface charge of phospholipid membranes composed of phosphatidylcholine and dicetylphosphate. However, whereas dibucaine decreases the ^{22}Na permeability of these structures, amiloride brings about an increase. The incorporation of cholesterol into such membranes normally reduces cation permeability, primarily through alterations in membrane viscosity. However, amiloride becomes even more effective in increasing ^{22}Na movement in cholesterol-rich membranes; dibucaine, rather than reducing sodium permeability, actually causes an increase under these circumstances. It is proposed that dibucaine is buried in the membrane with its positively charged end anchored at the interface. The resultant alteration in surface charge reduces cation movement. On the other hand, amiloride is probably confined to the interfacial region, bound there by the electrostatic attraction between its positively charged amidino group and the phosphate ion of the phospholipid. The exact means by which this agent increases cation permeability is unclear, but probably involves changes localized to the interface.

LOCAL ANESTHETICS block nerve conduction by interfering with the increased sodium conductance associated with the rising phase of the action potential.[1,2] In addition, these same agents can effectively reduce the cation permeability of model phospholipid bilayer membranes.[3] Since the phospholipids of biological membranes are also arranged in bilayer form,[4] the study of these model systems could conceivably give useful information on the action of these agents at the molecular level.

Bangham *et al.*[5] observed that cationic anesthetics could neutralize the negative surface charge of membranes composed of phosphatidylcholine and the long-chain anion, dicetylphosphate. This change in surface charge was associated with a reduction in the movement of ^{42}K across these same structures. This association was not surprising in view of the known relationship between surface charge and cation permeability previously described for this system.[6]

Structurally the useful local anesthetics are usually composed of a hydrophilic amino group connected by an intermediate chain to a lipophilic aromatic residue.[2] The structure of dibucaine is illustrated in Fig. 1. In this same figure, the structure

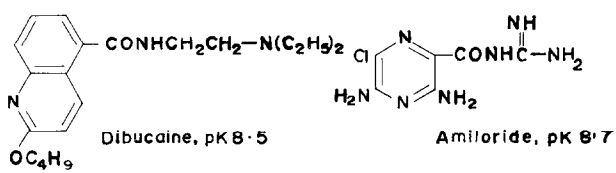


FIG. 1. Structures and pK values of the local anesthetic dibucaine and the diuretic agent amiloride.

of the diuretic agent, amiloride, is compared to that of dibucaine. The pK of these two molecules is approximately 8·5 (Fig. 1) so that both will be predominantly ionized (positive charge) at pH 7·5.

In view of their structural similarities, it was anticipated that amiloride might also decrease the negative surface charge of phosphatidylcholine–dicetylphosphate membranes and reduce cation permeability. Initial experiments indicated that amiloride did have the expected change on surface charge. However, in the presence of this agent, cation permeability was found to be increased rather than decreased. In view of their opposing effects on cation movement these two drugs must interact with the model membrane in quite dissimilar ways. The nature of these interactions is explored more fully in this paper.

MATERIALS AND METHODS

Materials. Phosphatidylcholine or lecithin was extracted from fresh eggs and purified by Alumina and silicic acid chromatography.[6] The phosphatidylcholine was stored as a chloroform solution under N_2 at $-20°$. Cholesterol and dicetylphosphate were obtained from Sigma Chemical Co., St. Louis Mo. Dibucaine was purchased from K & K Laboratories, Plainview, N.Y., whereas amiloride and its analogues were kindly supplied by Dr. W. D. Dorian of Merck, Sharp & Dohme Canada Ltd. ^{22}Na as the chloride salt was obtained from Amersham/Searle. All other chemicals were of reagent grade wherever possible. Twice-distilled water was used for all experiments.

Preparation of membranes. Since the preparation of lipid vesicles has been previously described,[7,8] only a brief outline will be presented. Appropriate aliquots of the chloroform solutions of phosphatidylcholine $\pm$ dicetylphosphate $\pm$ cholesterol were dried under vacuum in a glass tube. A small volume (approximately 1 ml/15 μmoles of lipid) of the required salt solution containing 2 μCi ^{22}Na was pipetted into the tube and the mixture mechanically shaken on a Rotamixer (at room temperature) until all of the lipid was suspended. The resulting milky dispersion was left overnight under N_2 at room temperature.

Each of the vesicles or liposomes, referred to above, consists of a series of concentric bimolecular phospholipid membranes. On the basis of theoretical considerations, it is probable that each bimolecular membrane forms an unbroken sheet,[6] thus creating within the liposome a series of discrete and isolated internal aqueous compartments. Several experimental observations support this contention. First, anions diffuse out of liposomes faster than cations.[6] Second, valinomycin has been shown to facilitate selectively the diffusion of potassium over sodium from liposomes containing equal concentrations of these two cations.[9] These observations strongly suggest that ion efflux out of these vesicles occurs across intact membranes. Furthermore, these observations would be very difficult to reconcile with any model depicting ion efflux as occurring through rupture of vesicles or via large aqueous channels across broken membranes.

The outermost membrane separates the whole vesicle from the continuous bulk aqueous phase. In the process of formation of these liposomes, solutes (e.g. ^{22}Na) originally present in the bulk aqueous phase will become sequestered within the internal aqueous compartments of the vesicles. The subsequent leakage of such solutes from these structures can be used as a measure of permeability of the phospholipid

bimolecular membranes. A fuller description of the liposome model system is given in several recent review articles.[9,10]

Measurement of ^{22}Na fluxes. The mechanics of measuring efflux rates in this system have been previously described.[8] Briefly, the lipid dispersion is passed down a column of Sephadex G-50 coarse (Pharmacia, Montreal) to remove excess tracer not trapped within the lipid vesicles.[6] One-ml portions (approximately 2 μmoles) of the eluted lipid are pipetted into dialysis bags which are dropped into stoppered glass tubes containing 10 ml of aqueous solution. After a 30-min "wash period," the bags are transferred to a new set of identical tubes for a 24-hr period. All tubes are placed in a shaking water bath maintained at 25°. The 24-hr period was chosen since the liposome displays a low leakage rate for trapped isotope and over this time interval sufficient counts appear in the external medium to give reproducible results.

The efflux kinetics for the liposome system have been described in a previous publication.[8] Briefly, these structures display first-order kinetics until about 50–80 per cent of initial trapped isotope has been lost.

At the termination of the 24-hr period, the ^{22}Na content of the various tubes and bags was measured on a Nuclear-Chicago gamma scintillation counter. Fluxes are expressed as the percentage of initial trapped radioactivity lost over the 24-hr time interval.

Electrophoretic measurements. Surface charges may arise from the presence of a net charge on the polar head group of the phospholipid or through selective adsorption onto the membrane surface of anions or cations from the bulk aqueous phase.

Phospholipid vesicles possessing surface charges will migrate under the influence of an electric field toward the electrode of opposite polarity. As each particle migrates, it carries with it a variable layer of counter ions and water molecules. The zeta potential represents the electric potential at the hydrodynamic slip plane of the lipid particle and under most circumstances will be less than the electrostatic potential at the actual membrane–water interface.[11] Within this limitation, the zeta potential can be used both as an approximate measure of the magnitude of the surface charge density and of any changes that it may undergo.*

The electrophoretic mobility of the lipid particles was measured at 25° in a cylindrical microelectrophoresis chamber.[12] The zeta potential was calculated according to the following relation:[13]

$$\xi = 12{\cdot}9\ V/E$$

where V = electrophoretic mobility in μsec^{-1}, E = applied voltage in V cm^{-1}, and ξ = the zeta potential in mV.

RESULTS

Figure 2, upper half, illustrates the effect of dibucaine, amiloride and calcium on the zeta potential of phosphatidylcholine–dicetylphosphate membranes. Amiloride and calcium appear to have equivalent effects on surface charge as measured by comparable reductions in zeta potential. Dibucaine actually causes a reversal of surface charge, the liposomes becoming positively charged at anesthetic concentrations in excess of 0·4 mM.

* The relationship between zeta potential and the electrostatic potential at the actual interface is schematically illustrated in Fig. 3–3, p. 115, of reference 13. The discrepancy between these two parameters is more pronounced in the presence of high surface charge densities.

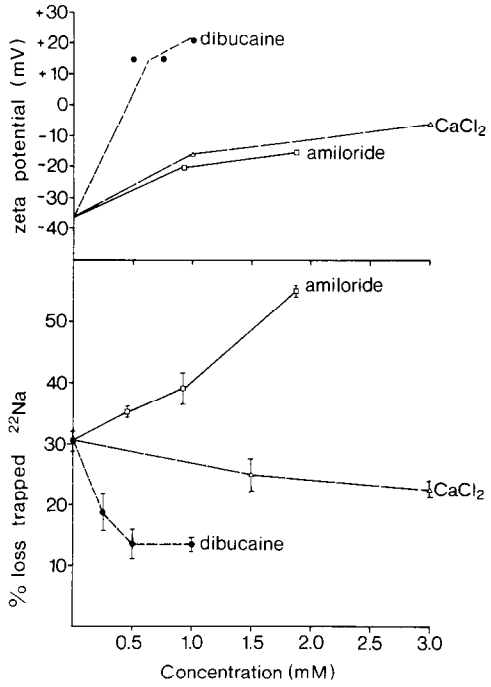


FIG. 2. Effects of calcium, dibucaine and amiloride on ^{22}Na efflux and zeta potential. Liposomes composed of 80 per cent phosphatidylcholine, 20 per cent dicetylphosphate (mole ratio) were formed and dispersed in 50 mM NaCl, 6 mM Tris (hydroxymethyl) aminomethane, pH 7·5. Dibucaine, $CaCl_2$ or amiloride was added to the bulk aqueous phase at the concentrations indicated on the abscissa. Experimental points represent means ± S.E. (n = 10 for $CaCl_2$, n = 20 for dibucaine and amiloride). The zeta potentials are the means of at least 20 measurements with the data being reproducible to within 10 per cent.

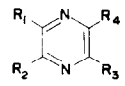
Despite their qualitatively similar effects on surface charge, these three cations have dissimilar effects on ^{22}Na efflux (Fig. 2, lower half). Calcium and dibucaine both decrease the rate of loss of ^{22}Na from these negatively charged liposomes, whereas amiloride brings about an increase.

Amiloride consists of a substituted pyrazine ring linked to a guanidine group. A series of analogues were tested to determine the structural features responsible for its properties. The design of these experiments was as follows. Liposomes composed of phosphatidylcholine–dicetylphosphate were formed in an NaCl solution. After elution from the Sephadex column, the lipid was divided into nine equal portions and pipetted into dialysis bags as described under Methods. Three of these portions served as a control in that the loss of trapped ^{22}Na was measured with the vesicles bathed by the NaCl solution only. Three portions were exposed to the salt solution containing 1·73 mM amiloride, while the remaining three samples were dialyzed against the salt solution containing an analogue of amiloride. In this way, the effects of amiloride and one of its analogues could be compared within the same lipid preparation. Comparison between lipid preparations was facilitated by arbitrarily assigning the control ^{22}Na efflux in each experiment the value one. Other effluxes were normalized to this value. The data are summarized in Table 1.

Guanidine, pyrazinamide and pyrazinecarboxylic acid have no effect on surface charge and little if any effect on ^{22}Na permeability. Examination of the properties of the other analogues leads to several general observations. First, small modifications in the substitutions on the pyrazine ring, such as replacement of the chlorine atom (NO1) or R_2 amino group (HO6) by hydrogen, markedly reduce the capacity of the resulting molecule to increase ^{22}Na efflux. Second, the two analogues (TO2, RO1) which are capable of increasing sodium permeability are also capable of decreasing surface charge. On the other hand, analogue GO2, which has a modification in the R_4 group only, possesses neither of these two properties. It would appear then, that although the capacity to decrease surface charge can be separated from the permeability effect (e.g. analogue NO1), the converse is not true. The three molecules which enhance ^{22}Na efflux (amiloride, TO2, RO1) all reduce the magnitude of the surface charge.

Tentatively it is proposed that the positively charged guanidine moiety functions to anchor the molecule electrostatically to the negatively charged membrane, allowing the ring portion to increase cation movement. This hypothesis is supported by the results of experiments using analogues as noted above and by two additional observations. First, when liposomes composed only of the neutral lipid phosphatidylcholine were exposed to amiloride, no significant increase in ^{22}Na permeability occurred. In this case, the lack of negative surface charge prevents the initial electrostatic interaction between amiloride and the membrane. Without this initial interaction, no increase in permeability is observed. Second, when both calcium and amiloride are present, ^{22}Na efflux is decreased, the membrane behaving as if calcium alone

TABLE 1. EFFECT OF ANALOGUES OF AMILORIDE ON ^{22}Na EFFLUX AND ZETA POTENTIAL*



Analogue	Concn (mM)	R_1	R_2	R_3	R_4	Normalized ^{22}Na efflux	$\Delta\xi$ (mV)
Amiloride	1·73	Cl	NH_2	NH_2	$CONHC(=NH)NH_2$	1·84 (1·54 2·3)	22
Inactive analogues							
Pyrazinamide	1·73	H	H	H	$CONH_2$	0·93 (0·77 1·17)	0
Pyrazinecarboxylic acid	1·73	H	H	H	COOH	1·08 (0·78 1·36)	0
L 590-154-00A01	1·73	H	H	NH_2	$CONHC(=NH)NH_2$	1·03 (1·02 1·04)	not tested
L 590-481-01H06	1·84	Cl	H	NH_2	$CONHC(=NH)NH_2$	0·95 (0·88 1·0)	not tested
L 593-791-01N01	1·50	H	NH_2	NH_2	$CONHC(=NH)NH_2$	1·10 (1·0 1·21)	22
L 591-260-01P06	1·62	Cl	H	NH_2	$CONHNHC(=NH)NH_2$	0·87 (0·8 0·93)	4
L 594-173-01G02	1·28	Cl	NH_2	NH_2	$CONHNHC(=NH)NH_2$	1·0 (0·85 1·16)	0
Guanidine	5					1·0 (0·98 1·01)	0
Active analogues							
L 591-605-01T02	1·45	Cl	$N(CH_3)_2$	NH_2	$CONHC(=NH)NH_2$	1·44 (1·38–1·51)	25
L 593-652-00R01	1·56	Cl	NH_2	NH_2	$CONHC(=NH)N(CH_3)_2$	1·37 (1·36 1·39)	12

* Liposomes composed of 80 per cent phosphatidylcholine, 20 per cent dicetylphosphate (mole ratio) were dispersed in 50 mM NaCl, 6 mM Tris (hydroxymethyl) aminomethane, pH 7·5. The lipid dispersion was divided into three portions to measure ^{22}Na efflux in the presence of NaCl only (control), NaCl plus 1·73 mM amiloride or NaCl plus an analogue (concentration given in table). The control efflux in each experiment was arbitrarily assigned the value one; other effluxes were normalized to this value. All experiments were performed in triplicate. The numbers for normalized effluxes represent means with the range of values given in parentheses. $\Delta\xi$ is the difference between the zeta potential of control liposomes (−36 mV) and the zeta potential of these same liposomes measured in the presence of amiloride or one of its analogues. In the text, the analogues beginning with an L are referred to by their last three letters (e.g. H06).

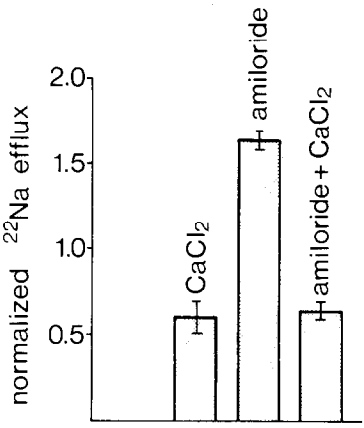


FIG. 3. Effect of $CaCl_2$ plus amiloride on ^{22}Na efflux. The experimental design was similar to that described in the footnote to Table 1. Liposomes composed of 80 per cent phosphatidylcholine, 20 per cent dicetylphosphate (mole ratio) were dispersed in 50 mM NaCl, 6 mM Tris (hydroxymethyl) aminomethane, pH 7·5. The dispersion was divided into four portions to measure ^{22}Na efflux in the presence of NaCl only (control), NaCl plus 1·73 mM amiloride, NaCl plus 3 mM $CaCl_2$, and NaCl plus 1·73 mM amiloride and 3 mM $CaCl_2$. The experiment was performed in triplicate. The control efflux was given the value one. Other effluxes were normalized to this value. The results illustrated represent means; error bars indicate the range of values.

were present (Fig. 3). The two cations calcium and amiloride, will both "compete" for negative sites on the membrane surface. Since ^{22}Na permeability is decreased when both agents are present, the calcium effect must be predominant. In terms of the proposed hypothesis, the calcium ions would prevent or at least significantly reduce the electrostatic binding of amiloride to the membrane, thus interfering with its effect on permeability.

It is possible that the non-ionized form of amiloride is responsible for the increased cation efflux. The observations already cited are probably inconsistent with this explanation, since the non-ionized species would be incapable of electrostatically binding to the membrane. Furthermore, the effect of amiloride was found to be enhanced by lowering the pH, a result opposite to that which would be expected if the non-ionized form was the active one. At pH 9·0 amiloride increased ^{22}Na efflux by a factor of 1·4 and by 2·5 times at pH 5·9.*

Alterations in the composition of the lipid vesicles have a striking influence on the properties of dibucaine and amiloride. Liposomes composed of 20 per cent dicetylphosphate (mole ratio) and various proportions of cholesterol and phosphatidylcholine were dispersed in either buffered sodium chloride solution (control), a sodium chloride solution containing amiloride, or a sodium chloride solution containing dibucaine. The results are illustrated in Fig. 4.

The ^{22}Na efflux rate in "control" liposomes decreases considerably as the membrane becomes enriched with cholesterol. The zeta potential of these vesicles, however, remains essentially unchanged, as noted in the upper half of Fig. 4. Dibucaine, which decreases ^{22}Na permeability in cholesterol-free membranes, actually increases ^{22}Na efflux as the proportion of cholesterol present in the vesicle increases. Incorpor-

* Phosphatidylcholine and dicetylphosphate themselves display no changes in ionization over this pH range.

ation of this sterol into the bilayer in excess of 20 per cent (mole ratio) also enhances the effect of amiloride on sodium permeability. The pattern illustrated in Fig. 4 appears quite complex. However, by replotting the data, as is done in Fig. 5, the influence of cholesterol becomes more apparent. The ^{22}Na efflux in the presence of sodium chloride only was assigned the value of one for each cholesterol concentration. Effluxes in the presence of the two pharmacologic agents were normalized to this value. In the absence of cholesterol, the efflux in the presence of dibucaine is about 0·5 of control, rising to a value eight times that of control at a sterol concentration of 45 per cent. Over the same cholesterol concentration range, amiloride increases ^{22}Na efflux from a value of 1·8 to approximately 22. Both of these agents, however, still reduce the negative surface charge of these cholesterol-rich vesicles, as measured by a fall in their zeta potential (Fig. 4), although the magnitude of this change is smaller than that which occurs with cholesterol-free liposomes.

DISCUSSION

Phosphatidylcholine is a zwitterion and forms lipid vesicles (or liposomes) which have an electrophoretic mobility of zero over a wide pH range. This lack of a measurable zeta potential has led to the conclusion that the ionic head group of phosphatidylcholine is co-planar, i.e. the phosphate and quaternary ammonium ions lie within

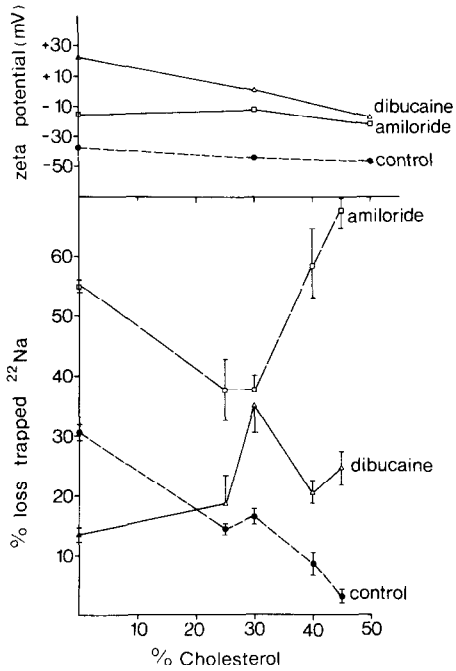


FIG. 4. Effects of dibucaine and amiloride on ^{22}Na efflux and zeta potential in the presence of cholesterol. Liposomes composed (by mole ratio) of 20 per cent dicetylphosphate, cholesterol (concentration indicated on abscissa) and phosphatidylcholine (to make 100 per cent) were dispersed in 50 mM NaCl, 6 mM Tris (hydroxymethyl) aminomethane, pH 7·5 (control). Amiloride or dibucaine was added to the bulk aqueous phase at a concentration of 1·73 mM and 1 mM respectively. Experimental points represent means ± S.E. (n = 6). The zeta potentials are the means of at least 20 measurements with the data being reproducible to within 10 per cent.

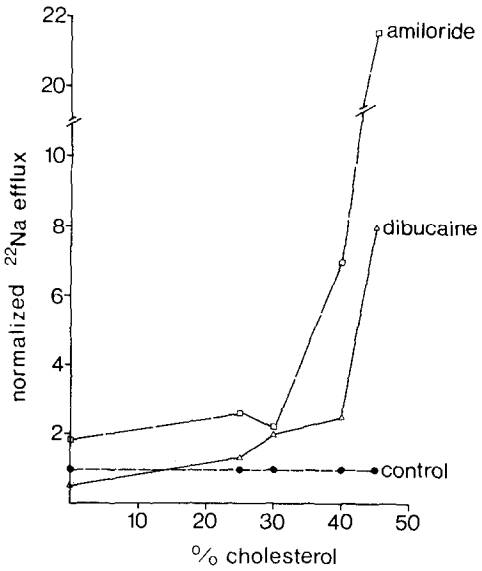


FIG. 5. The data of Fig. 4 were replotted as follows. For each cholesterol concentration, the control ^{22}Na efflux was given the value one. Effluxes in the presence of dibucaine or amiloride (at each cholesterol concentration) were normalized to this value.

the same plane normal to the membrane–solution interface.[10] Liposomes composed only of phosphatidylcholine display a low permeability to cations, as measured by the leakage rate of trapped radioactive markers.[8] A negative surface charge can be added to these vesicles by the incorporation of a long-chain anion such as dicetylphosphate into the bilayer structure. Such negatively charged vesicles are also more permeable to cations, leakage rates increasing as the surface charge density increases.[6] Such a relationship might be expected for several reasons. First, cations will accumulate in the electrical double layer adjacent to the negatively charged surface. This higher cation concentration will result in a larger flux across the membrane, which would be measured as an increased diffusion rate, since the bulk aqueous cation concentration would be unchanged. However, as Bangham *et al.* have noted,[6] the cation diffusion rate increases more with increments in surface charge density than can be accounted for by calculated changes in cation concentration adjacent to the interface. Second, the presence of a negative surface charge will increase the intermolecular electrostatic energy of repulsion, resulting in a greater membrane surface area and perhaps to a time average increase in the size of molecular "pores" existing normal to the plane of the interface. Agents which decrease the magnitude of the surface charge of phosphatidylcholine–dicetylphosphate vesicles should reduce cation permeability. Such is the case for calcium ions and the local anesthetic dibucaine (Figs. 2 and 3).

The effect of calcium is most likely due to the screening of surface charges by calcium ions present in the electrical double layer,[14] although a more specific binding of calcium ions to the phosphate group of dicetylphosphate cannot be entirely excluded. Dibucaine has a more pronounced effect than calcium on both surface

charge and cation permeability. In the presence of anesthetic concentrations exceeding 0·4 mM, liposomes demonstrate a reversal of charge, indicating a strong interaction between dibucaine and the membrane. Recently, Cerbon[15] has examined the interaction between tetracaine and purified phospholipids using NMR spectroscopy. The butylene "tail" of tetracaine is capable of penetrating a lipid membrane. The methyl terminal of this residue may reach the level of the seventh or eighth methylene group on the fatty acid chains of the phospholipids. Dibucaine also has a four-carbon tail at the nonpolar part of the molecule. In addition, it possesses an extra aromatic ring and, as Cerbon[15] suggests, should have a stronger hydrophobic interaction with a phospholipid membrane than tetracaine. By analogy with tetracaine, one might expect the methyl terminal of the butoxy group of dibucaine to also penetrate to the level of the seventh or eighth methylene group of the fatty acid chains. Figure 6 shows Corey–Pauling space-filling models of lecithin and dibucaine. When the anesthetic molecule is oriented parallel to the phospholipid with its tertiary amine group adjacent to the phosphate end of the lipid head group, then its methyl terminal does lie at about the level of the eighth methylene group of the fatty acid residue.

In view of the NMR spectroscopic data, one could postulate that dibucaine would penetrate the phosphatidylcholine–dicetylphosphate bilayer. The positively charged amine group of the anesthetic would lie in close proximity to the lipid head groups, while the remainder of the molecule would be buried within the membrane parallel to the fatty acid chains. An approaching cation would be electrostatically repelled by the positive surface potential generated by the tertiary amine group of the anesthetic. In this way, the cation diffusion rate across the membrane would be reduced.

Amiloride, like calcium and dibucaine, also reduces the magnitude of the negative surface potential of phosphatidylcholine–dicetylphosphate vesicles, but yet leads to an increase in cation permeability. Unfortunately, little information is available concerning the nature of the molecular interaction between amiloride and biomembranes. A space-filling model of this molecule is also illustrated in Fig. 6. Comparison with dibucaine leads to several observations. First, amiloride is only about 60 per cent as long as dibucaine and, if oriented parallel to lecithin (Fig. 6), would extend only to the level of the first or second methylene group of the fatty acid chain. Second, dibucaine possesses a more "hydrophobic" surface. Amiloride lacks both the second aromatic residue and the four-carbon tail of the anesthetic molecule. In addition, the chlorine atom attached to the pyrazine ring would probably be in a lower free energy state if kept within the water phase. As a tentative hypothesis, it is proposed that amiloride is largely confined to the interfacial region. The amidino group, bearing a positive charge, would lie close to a negatively charged head group, thus accounting for the reduction in surface potential. The remainder of the molecule, rather than penetrating into the hydrophobic interior of the membrane, like dibucaine, would also be confined to the interfacial region surrounding the lipid head groups. The predominant force holding the molecule in this region would be the electrostatic attraction between the positively charged amidino group and the phosphate (ion) of the phospholipid. By virtue of this electrostatic attraction, amiloride molecules would become concentrated at the bilayer interface. Analogues (Table 1) which do not decrease surface charge (i.e. are not electrostatically bound to the surface) would not be so concentrated. These analogues might be thought of as possessing a lower "partition coefficient" than amiloride between bilayer interface and water.

On the basis of this model, amiloride could conceivably increase cation permeability through several mechanisms. First, the presence of this molecule might disrupt water structure at the interface, which in turn might interfere with the packing arrangement of lipid head groups. Alternatively, a layer of oriented amiloride molecules might generate a dipolar potential such that the membrane interior became more negative with respect to the bulk aqueous phase. Such a dipolar field would increase the cation diffusion rate.[8] Unfortunately, the data are insufficient either to exclude these possibilities or to choose between them. Regardless of its mechanism of action, however, minor modifications in molecular structure such as removal of an amino group or chlorine atom can destroy its capacity to increase cation permeability (Table 1).

When cholesterol is incorporated into the bilayer structure, ^{22}Na efflux falls by an order of magnitude (Fig. 4). The presence of cholesterol in egg lecithin bilayers has been demonstrated to decrease membrane fluidity by increasing the degree of extension of the fatty acid chains and reducing the amplitude of motion of their long axes.[16] In addition to inducing these changes in hydrocarbon packing, cholesterol also alters the dipolar field at the membrane–solution interface such that the membrane interior becomes more positive with respect to the bulk aqueous phase.[17] Lipid bilayers* containing this sterol demonstrate a reduced permeability to anions,[18] Rb–valinomycin complex[19] and nonelectrolytes,[20] consistent with the changes in membrane "viscosity" and dipole potential noted above.

As illustrated in Figs. 4 and 5, dibucaine and amiloride actually increase the cation permeability of cholesterol-rich vesicles. As already discussed, dibucaine probably penetrates the membrane to a depth of the seventh or eighth methylene group of the fatty acid chains. Cholesterol is oriented in the bilayer with the 3β-hydroxyl group at the membrane–water interface. Furthermore, a space-filling model of this sterol indicates it has an overall length of 19 Å, comparable to the length of dibucaine (Fig. 6). Finally, Seeman[1] has demonstrated that anesthetics expand biomembranes some ten times more than can be accounted for by the actual volume occupied by the anesthetic molecules. Most of this expansion appears to be due to a "loosening" of interior membrane structure. In light of these observations and as a first approximation, it is proposed that the buried portion of dibucaine interferes with the hydrophobic interactions between cholesterol and the phospholipid fatty acid chains necessary for the observed sterol-induced changes in membrane fluidity.

The 3β-hydroxyl group has also been found to be an essential structural requirement for the lipid sterol interaction.[21] Furthermore, it appears that hydration of this group is important for this interaction.[21] If amiloride, as suggested, is largely confined to the interfacial region, it might very well disrupt proper hydration of this group, thus reducing the sterol-induced changes in membrane structure and increasing cation permeability.

It is of interest that the capacity of amiloride and dibucaine to increase ^{22}Na efflux becomes greatly enhanced when the cholesterol concentration (by mole ratio) exceeds 30 per cent (Fig. 5). Although the exact significance of this observation is

* Cholesterol has a rather complicated effect on hydrocarbon packing depending upon whether the phospholipid is above or below its transition temperature (Tc). Membranes composed of phospholipids below their Tc display an increase in permeability when cholesterol is added,[20] as a result of an increase in the mobility of their fatty acid chains.[16] Phospholipids above their Tc behave as already described for egg lecithin when cholesterol is added.

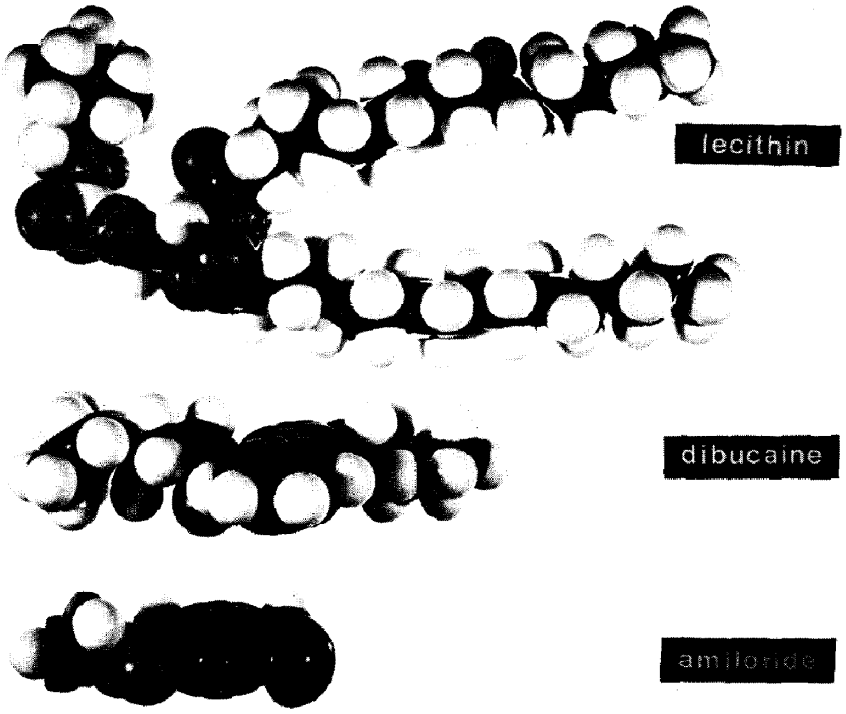


Fig. 6 Corey–Pauling space-filling models of lecithin (phosphatidylcholine), dibucaine and amiloride. For simplicity, two palmitic acid residues have been used to form the phospholipid. Lengths of these molecules are: lecithin, 28 Å; dibucaine, 19 Å; amiloride, 11·5 Å

unclear, it may indicate the occurrence of some type of structural rearrangement in the membrane when the mole ratio of cholesterol to phosphatidylcholine + dicetylphosphate exceeds 1:2. Lecuyer and Dervichian[22] have studied the structure of aqueous mixtures of egg phosphatidylcholine or lecithin and cholesterol by X-ray diffraction. The properties of this mixed system undergo a change when a cholesterol to lecithin mole ratio of 1:2 is reached. The measured long spacing, corresponding to the sum of the widths of the lipid and aqueous layers, goes through a maximum at this ratio and then decreases. The mechanism is as follows. Cholesterol initially causes an increase in the thickness of the hydrocarbon region due to "straightening" of the fatty acid chains. This phenomenon reaches a maximum at a cholesterol to lecithin mole ratio of 1:2. With greater proportions of cholesterol, the width of the apolar region remains relatively constant, but there is a subsequent decrease in the thickness of the water layers. Ladbrooke *et al.*[23] studied the effect of cholesterol on the gel to liquid crystal transition for several lipids. The addition of this sterol to dipalmitoyl lecithin in water causes an abrupt lowering of the transition temperature when the cholesterol concentration exceeds 20 moles/100%. No transition at all is observed with an equimolar ratio of lecithin and cholesterol.

Although the results of these two studies do not provide a ready explanation for the marked increase in slope in the curves of Fig. 5, they do indicate that the properties of lecithin–cholesterol membranes will display dramatic changes at a cholesterol to lecithin mole ratio of 1:2.

Many pharmacologic agents are thought to have their locus of action at the plasma membrane. The initial step is often viewed as some type of interaction between the drug and a membrane receptor. Such a receptor must have as one of its properties the capacity to discriminate between structurally similar molecules. It is of interest that simple phospholipid membranes can make such discriminations. Salicylate (*o*-hydroxybenzoate) and its analogue *p*-hydroxybenzoate behave quite differently in this system both in terms of their mechanism of permeation[18] and their capacity to increase ^{22}Na permeability.[8] Furthermore, lecithin membranes exposed to salicylate become negatively charged, indicating surface adsorption of this anion. This property is not possessed by *p*-hydroxybenzoate.[18] The results in Table 1 indicate that phosphatidylcholine–dicetylphosphate vesicles can discriminate between amiloride and structurally very similar analogues. Since phospholipids are important components of biological membranes, it is not unreasonable to expect that they will prove to be the actual "receptors" for many drugs.

Acknowledgements—This work was supported by the Medical Research Council of Canada. I would like to thank Mrs. Marlene Young for expert technical assistance.

REFERENCES

1. P. SEEMAN, *Pharmac. Rev.* **24,** 583 (1972).
2. J. M. RITCHIE, P. J. COHEN and R. D. DRIPPS, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. GOODMAN and A. GILMAN), p. 367. MacMillan, New York (1965).
3. D. PAPAHADJOPOULOS, *Biochim. biophys. Acta* **265,** 169 (1972).
4. S. J. SINGER, *Ann. N.Y. Acad. Sci.* **195,** 16 (1972).
5. A. D. BANGHAM, M. M. STANDISH and N. MILLER, *Nature, Lond.* **208,** 1295 (1965).
6. A. D. BANGHAM, M. M. STANDISH and J. C. WATKINS, *J. molec. Biol.* **13,** 238 (1965).
7. M. A. SINGER and A. D. BANGHAM, *Biochim. biophys. Acta* **241,** 687 (1971).
8. M. A. SINGER, *Can. J. Physiol. Pharmac.* **51,** 779 (1973).

9. A. D. BANGHAM, *Fedn. Eur. Biochem. Soc. Eighth Meeting*. Amsterdam, Vol. 28, p. 253. American Elsevier, New York (1972).
10. A. D. BANGHAM, *Prog. Biophys. molec. Biol.* **18,** 29 (1968).
11. D. A. HAYDON, *Recent Prog. surf. Sci.* **1,** 94 (1964).
12. A. D. BANGHAM, R. FLEMANS, D. H. HEARD and G. V. F. SEAMAN, *Nature, Lond.* **182,** 642 (1958).
13. J. T. DAVIES and E. K. RIDEAL, *Interfacial Phenomena*, p. 134. Academic Press, New York (1963).
14. S. G. A. MCLAUGHLIN, G. SZABO and G. EISENMAN, *J. gen. Physiol.* **58,** 667 (1971).
15. J. CERBON, *Biochim. biophys. Acta* **290,** 51 (1972).
16. S. SCHREIER-MUCCILLO, D. MARSH, H. DUGAS, H. SCHNEIDER and I. C. P. SMITH, *Chem. Phys. Lipids* **10,** 11 (1973).
17. G. SZABO, G. EISENMAN, S. G. A. MCLAUGHLIN and S. KRASNE, *Ann. N.Y. Acad. Sci.* **195,** 273 (1972).
18. M. A. SINGER, *Can. J. Physiol. Pharmac.* **51,** 523 (1973).
19. J. DEGIER, C. W. M. HAEST, J. G. MANDERSLOOT and L. L. M. VAN DEENEN, *Biochim. biophys. Acta* **211,** 373, (1970).
20. J. DEGIER, J. G. MANDERSLOOT and L. L. M. VAN DEENEN, *Biochim. biophys. Acta* **173,** 143 (1969).
21. B. DE KRUYFF, R. A. DEMEL, A. J. SLOTBOOM, L. L. M. VAN DEENEN and A. F. ROSENTHAL, *Biochim. biophys. Acta* **307,** 1 (1973).
22. H. LECUYER and D. G. DERVICHIAN, *J. molec. Biol.* **45,** 39 (1969).
23. B. D. LADBROOKE, R. M. WILLIAMS and D. CHAPMAN, *Biochim. biophys. Acta* **150,** 333 (1968).