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THE EFFECT OF AMINE STRUCTURE ON COMPLEXATION WITH LASALOCID IN MODEL MEMBRANE SYSTEMS

I. IDENTIFICATION OF CHARGED COMPLEXES IN LIPID BILAYER MEMBRANES

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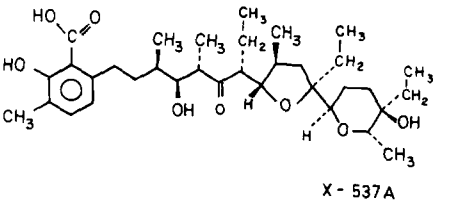
The electrical properties of X-537A (lasalocid) doped lipid bilayer membranes were studied in the presence of a series of nine biogenic amines which contain β-phenylethylamine as the basic structural unit. The ionophore antibiotic was found to form charged complexes within the membrane during the transport of some of the amines. The dependence of membrane conductance on the concentration of ionophore and amine was studied. The amines are divided into three classes according to the nature of the complexes formed: (1) charged complex involving two ionophores (phenylephrine, metanephrine, and amphetamine); (2) charged complex containing three ionophores (dopamine, norepinephrine and epinephrine); and (3) no charged species formed (*p*- and *m*-tyramine and β-phenylethylamine).

Introduction

Lasalocid, X-537A [1] (Fig. 1), is the smallest of the carboxylate antibiotics [2] and functions as an ionophore, or ion carrier, by forming specific complexes with a great variety of cations, rendering them lipophilic, and providing a means for their transport across apolar barriers. X-537A forms a shell by head-to-tail hydrogen bonding of the deprotonated carboxyl group with the hydroxyl group at the opposite end of the molecule; the ionophore then chelates the cation through ether, carbonyl and hydroxyl oxygens, resulting in a neutral complex with a hydrophobic exterior.

In addition to complexing monovalent cations [3–6], divalent cations [3–7] and amines in solution [8–11], X-537A also acts as an ion transporter in biological [12–16] and artificial lipid bilayer membranes [16–23]. The physiological effects associated with X-537A result mainly from its ability to complex with and transport Ca^{2+} and



X - 537A

Fig. 1. Structural formula of ionophore X-537A. The pK_a of the carboxylic acid is 3.7.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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catecholamines across lipid membranes [16,17]. Among a group of four structurally similar carboxylic ionophores, X-537A shows the greatest affinity for ethanolamine, norepinephrine and epinephrine [16].

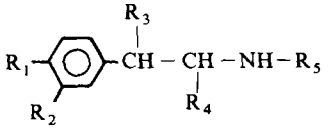
In the present study, ionophore-induced bilayer conductance under the influence of a series of nine biogenic amines was determined, systematically varying the type, number and position of substituents on the β-phenylethylamine structural unit (Table I).

At pH 7.0, at which the bilayer membrane electrical properties were monitored, the ionophore X-537A at the interface is negatively charged ($pK_a = 3.7$) [3] and the amines positively charged ($pK_a \sim 8.5$–10) [24] so that a 1:1 neutral or 'electrically silent' complex would be expected. However, it has been shown that a charged species is formed in the presence of dopamine [17]. Preliminary experiments indicated that not only did some (though not all) of the amines form charged complexes, but the nature of the complex depended on the structure of the amine.

The goals of this study were two-fold: (1) to determine the mechanism of action of X-537A in the transport of amines through bilayer membranes; and (2) to correlate amine structure with its ability to complex with the ionophore. This knowledge will contribute toward our understanding of the molecular basis for selective transport of amines and provide the basis for the investigation of ionophores as unique and specific drug delivery systems.

TABLE I

STRUCTURAL FORMULAE OF THE BIOGENIC AMINES



Amine	R_1	R_2	R_3	R_4	R_5
β-Phenylethylamine	H	H	H	H	H
Amphetamine	H	H	H	CH_3	H
p-Tyramine	OH	H	H	H	H
m-Tyramine	H	OH	H	H	H
Dopamine	OH	OH	H	H	H
Norepinephrine	OH	OH	OH	H	H
Epinephrine	OH	OH	OH	H	CH_3
Metanephrine	OH	OCH_3	OH	H	CH_3
Phenylephrine	H	OH	OH	H	CH_3

Materials and Methods

All chemicals (with the exception of lasalocid) were used without further purification. Lasalocid was obtained as the sodium salt (Aldrich, Milwaukee, WI) acidified with a slight molar excess of hydrochloric acid, and extracted into chloroform which was then evaporated to dryness to yield the free acid. The amines were purchased as the hydrochloride salts: dopamine, DL-norepinephrine, DL-metanephrine, L-phenylephrine, β-phenylethylamine (Sigma, St. Louis, MO); DL-epinephrine (ICN Pharmaceuticals, Plainview, NY); *p*-tyramine (Merck, Darmstadt, F.R.G. or Sigma); *m*-tyramine (Vega, Tucson, AZ). DL-Amphetamine was synthesized at the Shemyakin Institute of Bioorganic Chemistry (Moscow, U.S.S.R.); purity was determined by TLC. Racemic amines were used since X-537A does not seem capable of distinguishing between enantiomers in solution (unpublished data). L-α-Phosphatidylcholine (dioleoyl) was obtained from Serva (Heidelberg, F.R.G.).

All solvents were spectral grade. Distilled water was used in the preparation of buffer solutions.

The two compartment teflon chamber diagrammed in Fig. 2 was used for measuring conductance of the lipid bilayer membrane. The temperature was maintained at 25.0 ± 0.5°C by a continuous flow, constant temperature bath. Lipid bilayers were formed from a mixture of dioleoylphosphatidylcholine and cholesterol (2:1, w/w) in *n*-octane (1%w/v lipids) [25]. The bilayer membrane was formed across a hole (0.05 cm diameter) pierced in a 25 μm thick teflon partition, which had been fixed between the two compartments. The aqueous solution bathing the membrane (3.0 ml on each side) was buffered at pH 7.0 by Hepes or triethanolamine, where indicated (10.0 mM in each case); ascorbic acid (500 μM) was included to prevent amine oxidation.

Ionophore X-537A was dissolved directly in the membrane-forming lipid solution (10%v/v $CHCl_3$ in *n*-octane). Although the exact concentration of X-537A in the membrane was not known, it was assumed that it was proportional to the concentra-

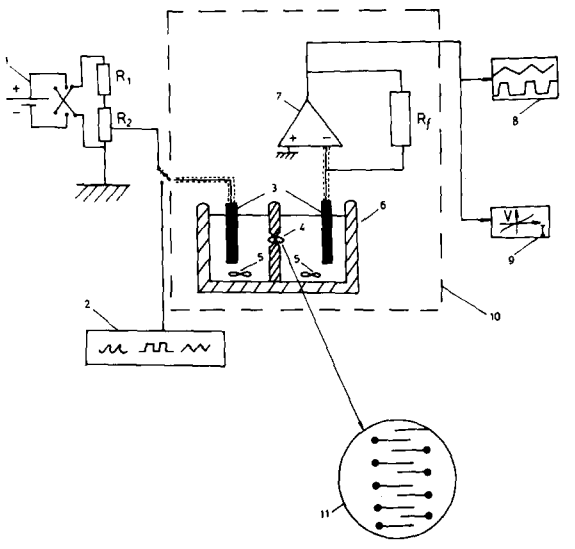


Fig. 2. Scheme of the experimental set-up for measuring the electrical characteristics of bilayer membranes. 1, battery; 2, function generator; 3, Ag/Ag^+Cl^- electrodes; 4, bilayer membrane; 5, magnetic stirrers; 6, thermostatically controlled teflon cell; 7, operational amplifier; 8, oscilloscope; 9, *X-Y* recorder; 10, shield; 11, schematic representation of the orientation of the lipid molecules in the bilayer.

tion in the lipid solution. Given the extremely low aqueous solubility of the ionophore (0.01%) and the fact that the current-voltage characteristics (conductance) did not change with time, it is unlikely that any significant amount partitioned into the aqueous phase.

For measurements of membrane conductance vs. ionophore concentration ($C_{X\text{-}537A}$), the amine concentration in the aqueous bathing solution on both sides of the membrane was 50.0 mM. For determinations of membrane conductance vs. amine concentrations (C_{amine}), the ionophore concentration in the lipid solution was 3.5 mM.

For each membrane the raw value for membrane conductance was multiplied by a factor C_0/C_i to account for differences in membrane area due to the small torus at the membrane edges (where C_0 is the maximum observed capacitance for a set of membranes under identical conditions and C_i is the capacitance for a particular membrane).

A 60 mV potential was applied across the membrane and conductance (i.e., the reciprocal of the resistance) was measured. Membrane conductance in the absence of ionophore was between $1 \cdot 10^{-8}$ and $6 \cdot 10^{-8}$ mho $\cdot$ cm^{-2}.

The fluorescence emission intensity of X-537A in *n*-octane (containing 1%v/v MeOH) was measured at $\lambda_{ex} = 310$ nm and $\lambda_{em} = 420$ nm.

The absorbance of the ionophore was measured in *n*-octane at two concentrations: 3.97 μM and 10.4 μM, at $\lambda = 310$ nm.

Circular dichroism measurements of X-537A were carried out in *n*-octane and 1-octanol. $\Delta\epsilon$, the difference of the molar decadic coefficients, was calculated from the following equation:

$$\Delta\epsilon = \frac{Sn}{C_{X\text{-}537A}l}$$

where $\Delta\epsilon = \epsilon_l - \epsilon_r$ and subscripts refer to left-handed and right-handed circularly polarized light. *S*, sensitivity, *n*, height of peak maximum and *l*, cell path length, are experimentally determined parameters.

Results and Discussion

Specific membrane conductance for the series of amines shown in Table II (present at a concentration of 50.0 mM on both sides of the membrane) was calculated using Ohm's Law and plotted as a function of the concentration of ionophore in the membrane-forming lipid solution (Fig. 3A–C). Membrane conductance (g_0) is related to ionophore concentration by the equation $g_0 = aC^{\alpha}_{X\text{-}537A}$ (where α is the molecularity of ionophore in the complex and '*a*' is a constant). α was determined by linear regression analysis of plots of log g_0 vs. log $C_{X\text{-}537A}$ (Table II). Theoretically, in the absence of charged species, membrane conductance should be independent of ionophore concentration; i.e., there should be no increase in membrane conductance.

Membrane conductance of the catecholamines, dopamine, norepinephrine and epinephrine (Fig. 3A), exhibit a third order dependence on ionophore concentration (slope, $\alpha = 2.9 \pm 0.1$) indicating that a charged species is formed containing three ionophore molecules. For metanephrine, phenylephrine and amphetamine (Fig. 3B) the slope, α, of the linear portion of the plot is approx. 2 (2.1 ± 0.1), suggesting a charged species which contains two ionophores.

TABLE II

REGRESSION ANALYSIS PARAMETERS FOR THE POWER CURVE $g_0 = aC^{\alpha}_{X\text{-}537A}$

n is the number of data points used to fit the curve, a is a constant of proportionality, α is the order of dependence of membrane conductance on ionophore concentration, and r^2 is the regression coefficient.

Amine	n	a	Slope, α	r^2
Dopamine	28	683.2	2.96	0.972
Norepinephrine	12	54.1	2.70	0.986
Epinephrine	12	26.0	2.90	0.988
Metanephrine	29	0.5	2.10	0.963
Phenylephrine	17	2.1	2.20	0.988
Amphetamine	13	0.1	2.01	0.964
Buffer(Hepes or triethanolamine)	30	$2.9 \cdot 10^{-5}$	0.88	0.918

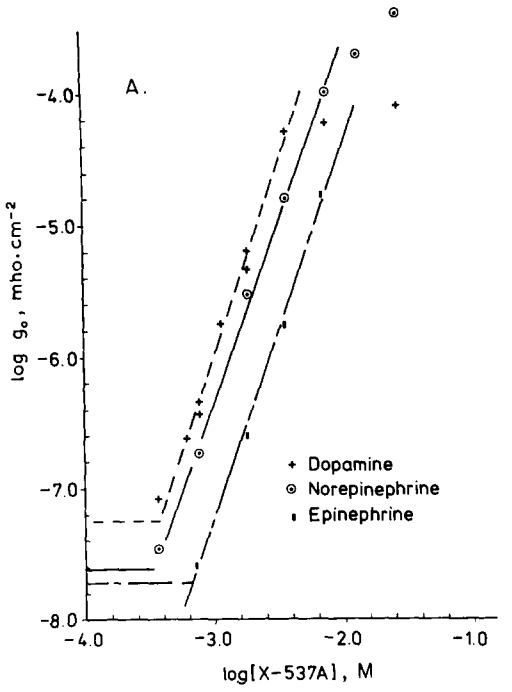
Previous studies have suggested that X-537A may be capable of dimerizing within a membrane when the pH of the bathing solution is close to the pK_a of the ionophore [18]. Several physical properties of the ionophore were measured in the absence of amine to establish the state of association of X-537A in the concentration range and under the same pH conditions used for conductance measurements. *n*-Octane and 1-octanol were chosen as solvents in an attempt to mimic the polarity of the membrane interior and exterior, respectively.

Fig. 4 shows a plot of X-537A fluoresence intensity, F, as a function of ionophore concentration in *n*-octane. If no change in the state of molecules in solution occurs, F should increase linearly with concentration; this behavior was confirmed at low concentrations of ionophore. The plot deviates from linearity for ionophore concentrations greater than 4 μM. The negatve deviation above this concentration appears to result from association of two or more ionophores since intermolecular interaction between the phenol rings (responsible for the fluorescent properties) in forming an aggregate quenches fluorescence [26]. Likewise, the absorbance ($\lambda = 310$ nm) of X-537A solutions did not vary linearly with ionophore concentration, supporting the hypothesis of ionophore self-association at higher concentrations. The apparent increase in optical density with concentration is surprising and no explanation is immediately apparent.

Self-association is confirmed by analysis of the CD spectra of X-537A. Spectra of X-537A in *n*-octane and 1-octanol are shown in Fig. 5. One chromophore ($\lambda_{max} \cong 290$ nm) results from the phenolic group and the second ($\lambda_{max} \cong 245$ nm) is attributed to the carboxylate group [18]. In both solvents, $-\Delta\epsilon$ for the two maxima decreases with increasing ionophore concentration (Fig. 6A,B) reflecting a change in the orientation of molecules in solution. In *n*-octane, the decrease in $-\Delta\epsilon$ is a linear function of ionophore concentration (Fig. 6A) whereas in 1-octanol $-\Delta\epsilon$ decreases, then reaches a plateau which indicates that no further association is occurring, i.e., the relative concentration of species is not changing with total concentration.

The minimum concentration of X-537A required to increase the membrane conductance in the presence of amine above that for the undoped membrane was determined. The point of intersection of the straight line portion of the double log plot in Fig. 3A,B with the horizontal line representing the bare membrane conductance was used to evaluate this ionophore concentration. For the series of amines shown in Table I it increases in the order dopamine < norepinephrine < phenylephrine < metanephrine < epinephrine < amphetamine. This order may reflect the selectivity of ionophore for the amines.

In the absence of amine (buffer at pH 7.0, Fig. 3C) the dependence of conductance on ionophore concentration is close to first order because the dissociated form of the weakly acidic ionophore itself is membrane soluble and contributes a background conductance [27]. *p*-Tyramine, *m*-tyramine and β-phenylethylamine inhibited conductance, including that attributed to $X\text{-}537A^-$. Inhibition of conductance may reflect the amine either complexing with the ionophore exclusively as a 1:1



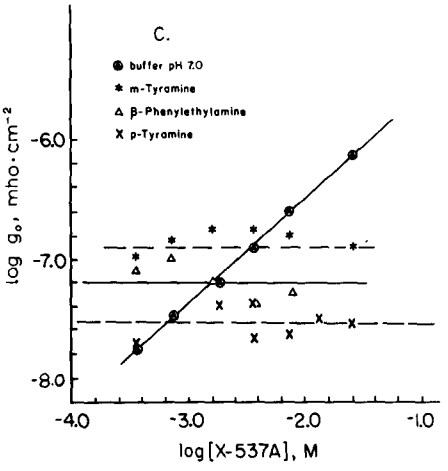
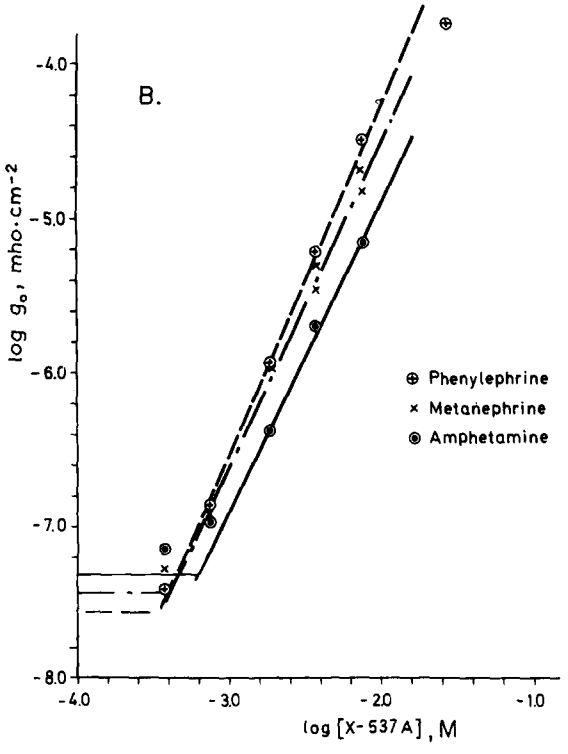


Fig. 3. Relation between specific membrane conductance, g_0 (mho·cm^{-2}) and concentration of ionophore X-537A in the membrane-forming lipid solution (mol·l^{-1}). pH of the aqueous phase bathing the membrane was buffered at 7.0 by Hepes (10.0 mM) and amine concentration on both sides of the membrane was 50.0 mM (10.0 mM in the case of epinephrine). Each data point represents an average for three to five membranes. The initial portions of the graphs represent the membrane conductance in the absence of ionophore. (C) The aqueous buffer was either 10.0 mM Hepes or 10.0 mM triethanolamine at pH 7.0.



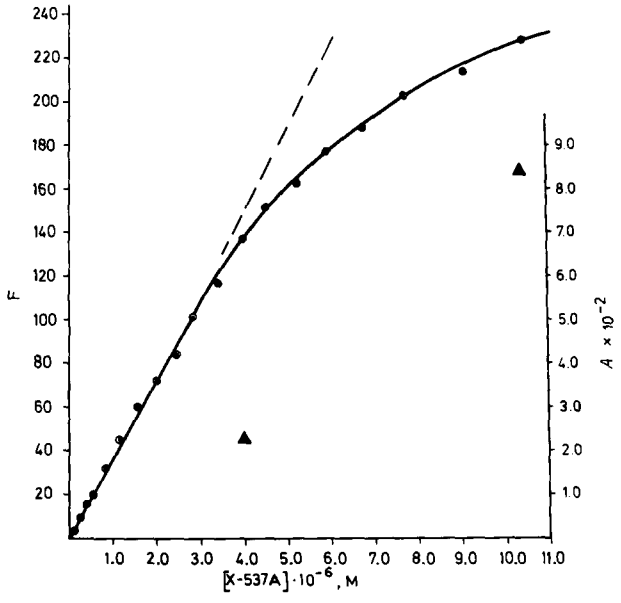


Fig. 4. Change in fluorescence intensity (F) as a function of ionophore concentration (mol·l^{-1}) in n-octane. $\lambda_{em}=420$ nm. Dotted lines represent theoretically expected fluorescence. Absorbance $A(\Delta)$ was measured at ionophore concentrations of 4 μM and 10.4 μM.

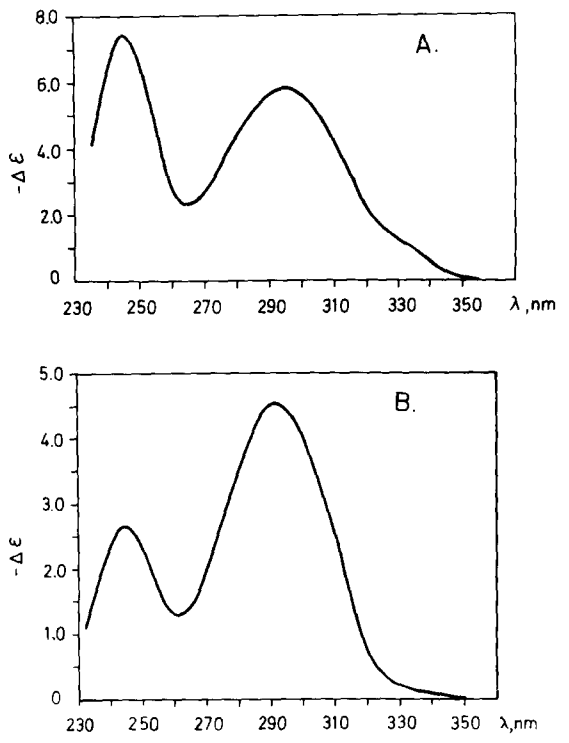


Fig. 5. CD spectra of X-537A. (A) $C_{X\text{-}537A} = 21.9$ μM in *n*-octane. (B) $C_{X\text{-}537A} = 164$ μM in 1-octanol. Conditions as described in Materials and Methods.

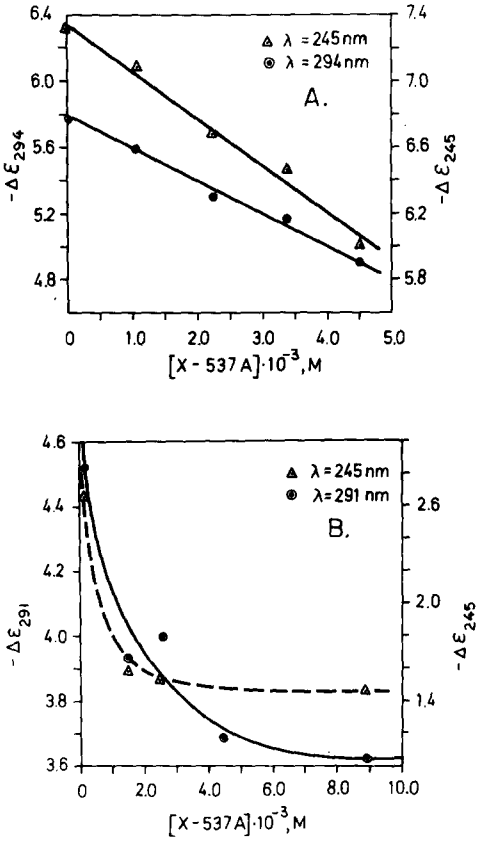
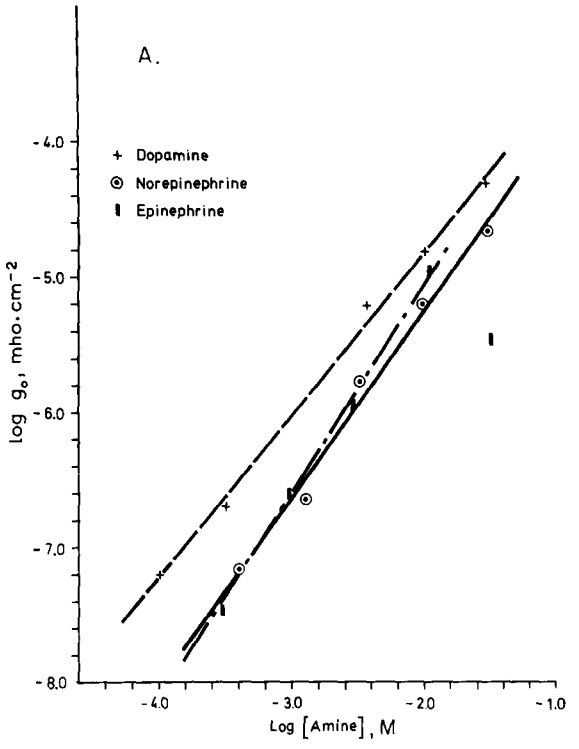


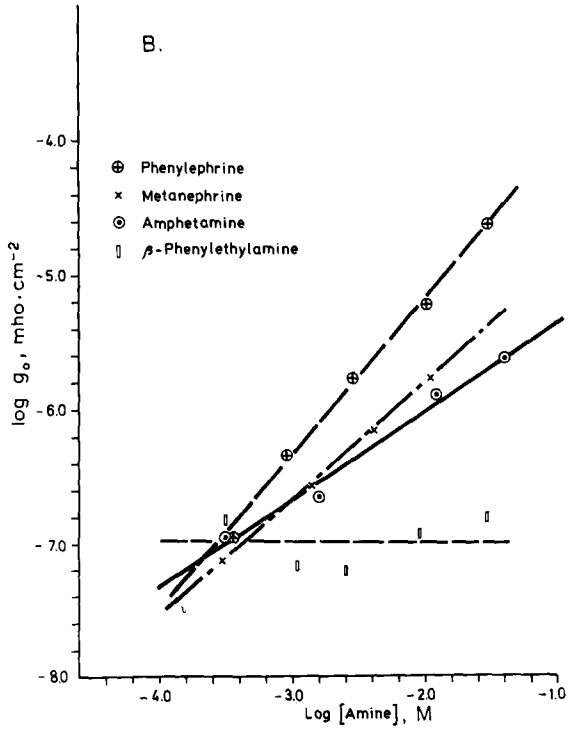
Fig. 6. Change in intensity of CD maxima ($-\Delta\epsilon$) as a function of ionophore concentration (mol·l^{-1}). (A) *n*-Octane as solvent: $\lambda_{max} = 245$ nm, △; $\lambda_{max} = 294$ nm; ⊙. (B) 1-Octanol as solvent: $\lambda_{max} = 245$ nm, △; $\lambda_{max} = 291$ nm, ⊙.

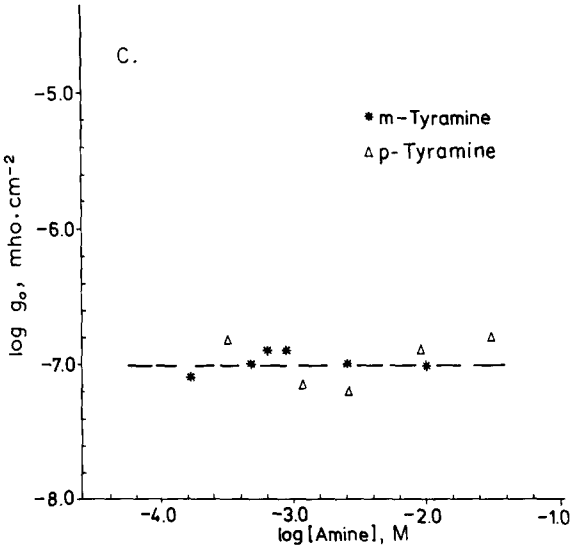
'electrically-silent' complex or possessing such a large complexation constant with the ionophore that binding of the amine at one aqueous-membrane interface is not followed by dissociation at the opposite interface.

To determine the number of amines involved in the charged complex, the dependence of membrane conductance on amine concentration (with amine present on both sides of the membrane) was plotted (Fig. 7 A–C). Membrane conductance is related to amine concentration by the equation $g_0 = bC^{\beta}_{amine}$ (where g_0 is conductance, β is the molecularity of amine in the complex and b is a constant). β was determined by regression analysis of the linear portions of plots of log g_0 vs. log C_{amine} (Table III). For the catecholamines, phenylephrine, metanephrine and amphetamine (Fig. 7A,B) log membrane conductance plotted vs. log amine concentration yielded straight lines with slope, β, of 1 (within experimental error). The deviation from first order dependence of membrane conductance on amine concentration for amphetamine ($\beta = 0.66$) and epinephrine ($\beta = 1.62$) can be traced to the inaccuracy in conductance measurements for amines with very large or very small complexation constants. β-Phenylethylamine and the tyramines do not produce an increase in membrane conductance at any concentration of amine studied (315 μM–31.5 mM) (Fig. 7 B,C) as expected if no charged species is formed.

The interaction of biogenic amines with X-537A across a lipid bilayer membrane is a complex, multicomponent process. Hence, a simple relationship correlating the structural features of an amine with its complexing characteristics is not expected. In the present study there is no clear definition between amine structure and the nature of the







Fg. 7. Relation between specific membrane conductance, g_0 (mho·cm^{-2}), and amine concentration (mol·l^{-1}) on both sides of the membrane. pH of the aqueous solution bathing the membrane was buffered at 7.0 by Hepes (10.0 mM) and ionophore concentration in the membrane-forming lipid solution was 3.5 mM. Each data point represents the average measurement for three to five membranes.

charged species which is detected. However, the following observations can be made.

For some amines a charged ionophore-amine complex is formed in the membrane with a stoichiometry of two or three ionophores to one amine. Amines in the first category (phenylephrine, metanephrine and amphetamine) contain

TABLE III

REGRESSION ANALYSIS PARAMETERS FOR THE POWER CURVE $g_0 = bC_{amine}^{\beta}$

n is the number of data points used to fit the curve, b is a constant of proportionality, β is the order of dependence of membrane conductance on amine concentration, and r^2 is the coefficient of regression.

Amine	n	b	Slope, β	r^2
Dopamine	11	$3.70 \cdot 10^{-3}$	1.19	0.972
Norepinephrine	15	$1.55 \cdot 10^{-3}$	1.27	0.950
Epinephrine	11	$1.71 \cdot 10^{-2}$	1.62	0.974
Metanephrine	13	$1.3 \cdot 10^{-4}$	0.94	0.941
Phenylephrine	18	$1.65 \cdot 10^{-3}$	1.17	0.965
Amphetamine	15	$1.85 \cdot 10^{-5}$	0.66	0.919

a methyl substituent on or adjacent to the terminal nitrogen. Charged complexes involving three ionophores (as observed for dopamine, norepinephrine and epinephrine) share the catechol moiety. No charged species was formed for β-phenylethylamine and the tyramines which lack substituents or contain a single hydroxyl substituent on the phenyl ring.

In summary the results of the present investigation show that in the absence of amine the ionophore self-associates in hydrophobic media; in the presence of some amines (dopamine, norepinephrine, epinephrine, phenylephrine, metanephrine, amphetamine) charged complexes are formed in bilayer membranes, the stoichiometry differing for different amines, whereas for *p*-tyramine, *m*-tyramine and β-phenylethylamine no charged complexes are formed.

Unpublished results from these laboratories show that these charged complexes represent only a small fraction of total amine flux. However, they may in fact be responsible for the electrical properties of biomembranes with a low intrinsic conductance.

Acknowledgements

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