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

II. IONOPHORE SELECTIVITY FOR AMINES IN LIPID BILAYERS AND AT OIL/WATER INTERFACES

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The ionophore antibiotic X-537A (lasalocid) transports biogenic amines across biological and artificial membranes. The major portion of amine flux (greater than 99%) occurs as a 1:1 neutral complex. The rank order of ionophore selectivity was determined for lipid bilayer membrane transport of amines based on a comparison of permeability coefficients: *p*-tyramine ~ β-phenylethylamine ~ amphetamine > methamphetamine > dopamine > phenylephrine ~ metanephrine > norepinephrine > epinephrine. This rank order is in agreement with results obtained from partitioning measurements which were carried out in parallel to the bilayer membrane experiments. A correlation between amine structure and binding characteristics has been developed.

Introduction

The ability of carboxylic ionophore X-537A (lasalocid) to induce changes in membrane permeability toward biogenic amines has been reported previously [1–7]. X-537A acts as a carrier ionophore, in contrast to channel- and sandwich-forming complexones [8]. The proposed general mechanism of transport consists of four steps [9]: (1) formation of the carrier-cation complex at the membrane/solution interface; (2) transport of the complex into and through the membrane; (3) release of the cation at the opposite interface; and (4) return of the ionophore to complete the cycle.

It has been shown previously [11] that X-537A forms charged complexes with some amines in lipid bilayer membranes. In the present study we examine the binding characteristics of the ionophore by comparing its interaction and transport properties with a series of substituted β-phenylethylamines at two types of interfaces commonly used to simulate the biomembrane: an aqueous/organic phase boundary, and a lipid bilayer membrane.

At an aqueous/organic phase boundary, partitioning of the amine in the presence of ionophore takes into account the heterogeneous complexation reaction at the interface as well as desolvation required for the amine to migrate into the less polar organic phase. The lipid bilayer membrane provides a more complex model for transport across biomembranes devoid of lipid-protein interactions.

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Materials and Methods

Amines were purchased as their hydrochloride salts and used without further purification: dopamine, DL-norepinephrine, DL-metanephrine, L-phenylephrine (Sigma, St. Louis, MO); DL-epinephrine (ICN Pharmaceuticals, Plainview, NY); *p*-tyramine (Merck, Darmstadt, F.R.G. or Sigma). DL-Amphetamine was synthesized at the Shemyakin Institute of Bioorganic Chemistry (Moscow, U.S.S.R.), purity was determined by TLC. Methamphetamine was synthesized by reacting phenylacetone with methylammonium chloride in the presence of sodium cyanoborohydride [11]. [^{3}H]Amphetamine, [^{3}H]dopamine, [^{3}H]norepinephrine and [^{3}H]epinephrine were purchased from New England Nuclear (Boston, MA). [^{3}H]Phenylephrine, [^{3}H]metanephrine, *p*-[^{3}H]tyramine and β-[^{3}H]phenylethylamine were synthesized at the Institute of Molecular Genetics (Moscow, U.S.S.R.). [^{3}H]Methamphetamine was prepared by first reacting phenylacetone with tritium oxide, then proceeding as for unlabeled amine. L-α-Phosphatidylcholine (dioleoyl) was obtained from Serva (Heidelberg, F.R.G.). Lasalocid (X-537A) was purchased as the sodium salt (Aldrich, Milwaukee, WI) and was converted to the free acid as described previously [10].

Distilled water was used in the preparation of aqueous buffer solutions, except in monolayer studies; for these, solutions were prepared with double distilled water having a specific conductance of not greater than 10^{-5} cm · mho. All other solvents were spectral grade.

Partition measurements. Partitioning of amines in the presence and absence of ionophore was measured between an aqueous buffer (pH 7.0, 10.0 mM Hepes) and a water saturated organic phase (1-octanol and *i*-octane mixtures), 15 ml each in 50 ml centrifuge tubes. The aqueous phase contained a constant concentration of amine and a small amount of tritiated amine as a tracer. A concentrated methanolic solution of ionophore was added (not more than 50 μl) to bring the final ionophore concentration to $1 \cdot 10^{-6}$–$4 \cdot 10^{-5}$ M and the tubes were shaken vigorously for at least 10 min at room temperature. Various shaking times were tested to ensure that equilibrium transfer of amine was complete after this time. After centrifuging, duplicate samples from each phase were counted in a Beckman Scintillation Counter.

Lipid bilayer studies. Lipid bilayer membranes were formed as described previously according to the method of Mueller [11,12]. The hole across which the bilayer membrane was formed was slightly larger (0.1 cm diameter) than that previously described to allow data collection for up to 1.5 h for transport studies. Ascorbic acid (0.5 mM) was included in the aqueous solution bathing the membrane to prevent oxidation of the amines.

Amine fluxes were monitored either radiochemically or fluorimetrically. For radiochemical monitoring, the membrane was formed with amine added to both compartments at a concentration of 50.0 mM. The resulting membrane containing X-537A (3.5 mM) was allowed to stand for at least 5 min. [^{3}]Amine (50 μl, $6 \cdot 10^6$ cpm) was then added to the donor compartment. After 10 min, 100 μl samples were withdrawn every 3 min from the receptor compartment and replaced by 100 μl of buffer solution containing unlabeled amine (50.0 mM).

To measure amine flux fluorimetrically, the membrane was formed in the absence of amine ($C_{X\text{-}537A} = 3.5$ mM), with 3.0 ml of aqueous buffer on each side of the barrier. After allowing 10 min for equilibration, 0.5 ml of a stock amine solution (0.07 M) was added to the donor compartment (10.0 mM final amine concentration) as the same volume (0.5 ml) of buffer solution was added simultaneously to the receptor side ($t = 0$ min). After 15–25 min, 100-μl samples were taken at regular intervals (replaced by buffer solution), diluted to 1.0 ml and analyzed fluorimetrically. Fluorescence was measured at the following wavelengths: dopamine, norepinephrine, epinephrine and metanephrine, $\lambda_{ex} = 280$ nm, $\lambda_{em} = 320$ nm; phenylephrine, $\lambda_{ex} = 264$ nm, $\lambda_{em} = 315$ nm; *p*-tyramine, $\lambda_{ex} = 270$ nm, $\lambda_{em} = 300$ nm. Amine concentrations were calculated from calibration curves, corrections being made for dilution.

Results and Discussion

The binding properties of ionophore X-537A in the presence of amines were measured and compared for X-537A lipid bilayer membranes and oil/water systems.

Partitioning

Partitioning of the amines between two liquid phases in the presence and absence of ionophore, was monitored by a radioisotope technique. A modification of the method of Lee et al. [13] was used to derive an equation to determine the stoichiometry of the ionophore-amine complex:

$$\log(\Delta K) = N \log[X^-]_{org} + \log K_C$$

where ΔK represents the increase in the partition coefficient of amine in the presence of ionophore; N is the stoichiometry of the ionophore-amine complex; $[X^-]$ is the ionophore concentration; and K_C is the complexation constant for ionophore with amine at the interface. This relationship assumes that: (1) complexation of ionophore with amine occurs only at the aqueous/organic interface; (2) at pH 7.0, the concentration of free base is negligible compared to that of the protonated amine; and (3) the ionophore is present only in the organic phase.

A double-logarithmic plot of ΔK vs. $[X^-]$ yields a straight line with slope N (defining the stoichiometry of the complex) and intercept log K_C as shown for amphetamine in Fig. 1. For all amines studied, N was approximately one (Table I) suggesting that the ionophore primarily forms 1 : 1

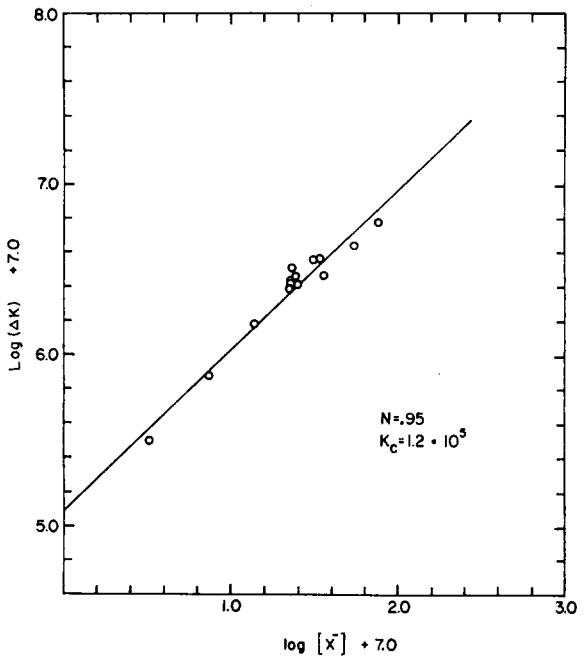

Fig. 1. Determination of K_c and stoichiometry of ionophore-amphetamine complex from a double log plot based on the equation $\log(\Delta K) = N \log[X^-] + \log(K_c)$. A linear regression analysis gives slope, N, the stoichiometry of the ionophore-amine complex at the aqueous/organic interface, and intercept, K_c, the complexation constant.

TABLE I

BINDING PARAMETERS FOR THE IONOPHORE-AMINE INTERACTION AT TWO INTERFACES

Amine	N [a]	K_c [b] (M^{-1})	P [c] ($cm \cdot s^{-1}$)
p-Tyramine	0.99±0.03	$(1.6\pm0.2)\cdot10^4$	$2.2\cdot10^{-4}$
β-Phenylethylamine	0.87±0.05	$(7.9\pm0.3)\cdot10^5$	$1.4\cdot10^{-4}$
Amphetamine	0.95±0.05	$(5.1\pm0.3)\cdot10^4$	$1.3\cdot10^{-4}$
Methamphetamine	0.93±0.05	$(3.5\pm0.2)\cdot10^3$	$8.8\cdot10^{-5}$
Dopamine	0.95±0.04	$(4.4\pm0.2)\cdot10^3$	$1.2\cdot10^{-4}$
Phenylephrine			$8.4\cdot10^{-5}$
Metanephrine	1.03±0.04	$(2.1\pm0.3)\cdot10^3$	$9.0\cdot10^{-5}$
Norepinephrine	1.04±0.07	$(6.0\pm0.5)\cdot10^2$	$4.0\cdot10^{-5}$
Epinephrine	1.2 ±0.08	$(1.3\pm0.2)\cdot10^2$	$1.4\cdot10^{-5}$

[a] Determined from the slope of double log plots of ΔK vs. $[X^-]_{org}$ according to the following equation (terms as defined in text): $\log(\Delta K) = N \log[X^-]_{org} + \log(K_c)$; average ±S.D. for four to six determinations.

[b] Complex formation constants, K_c for the ionophore-amine complex at an aqueous/1-octanol boundary were determined as described in Fig. 3; average ±S.D. for four to six determinations.

[c] Permeability coefficients, P, were calculated from $J = P \cdot \Delta C$ where J is the unidirectional amine flux across an X-537A doped bilayer membrane and ΔC is the concentration difference across the membrane; flux was measured either by using a [^{3}H]amine tracer or by fluorescence (Table II).

complexes with amines. Such complexes would be expected to be electrically-neutral since at pH 7.0, the amines are protonated ($pK_a \sim 8.5-1.0$) [14] and at the interface the ionophore is negatively charged ($pK_a = 3.7$) [2,15].

Complexation constants, K_C, for five amines were also determined as a function of organic phase composition (Fig. 2) by increasing the mole fraction of 1-octanol in *i*-octanol/1-octanol mixtures. It has been suggested that solvation of amines through hydrogen bonding reduces the extent of complexation, as it is presumed that desolvation of the amine in the aqueous phase must occur before it can complex with the ionophore [16]. Since amphetamine and methamphetamine have no hydroxyl groups to interact with the aqueous phase, complexation by the ionophore is expected to be more energetically favored than for other amines in the series with extensive hydroxyl group substitution. Dopamine, norepinephrine and epinephrine, with two or three hydroxyl substituents would be expected to exhibit lower values of K_C because of the opportunity for extensive solvation of the amine through hydrogen bonding in the aqueous phase. Such trends were observed (Fig. 2A and B). A comparison of the ability of X-537A to complex a primary versus a secondary amine can also be made. The decreased affinity of the ionophore for methamphetamine and epinephrine, relative to amphetamine and norepinephrine, respectively, appears to be due to steric hindrance by the *N*-methyl substituent diminishing electrostatic interaction between the protonated amine and the ionophore carboxylate.

The polarity of the organic phase was varied to mimic changes in the polarity of the membrane that take place in progressing from the polar interface to the apolar interior by incremental additions of octanol to a hydrocarbon phase. For amines with no hydrogen bonding capabilities beyond the terminal nitrogen (amphetamine and methamphetamine, Fig. 2A) K_C decreases as the fraction of octanol is increased, although with methamphetamine a slight increase in K_C is observed as the composition approaches that of pure octanol. Increased solute-solvent interaction in octanol manifests itself as an increased solubility of ionophore. This increased interaction with solvent may be responsible for a decrease in interaction

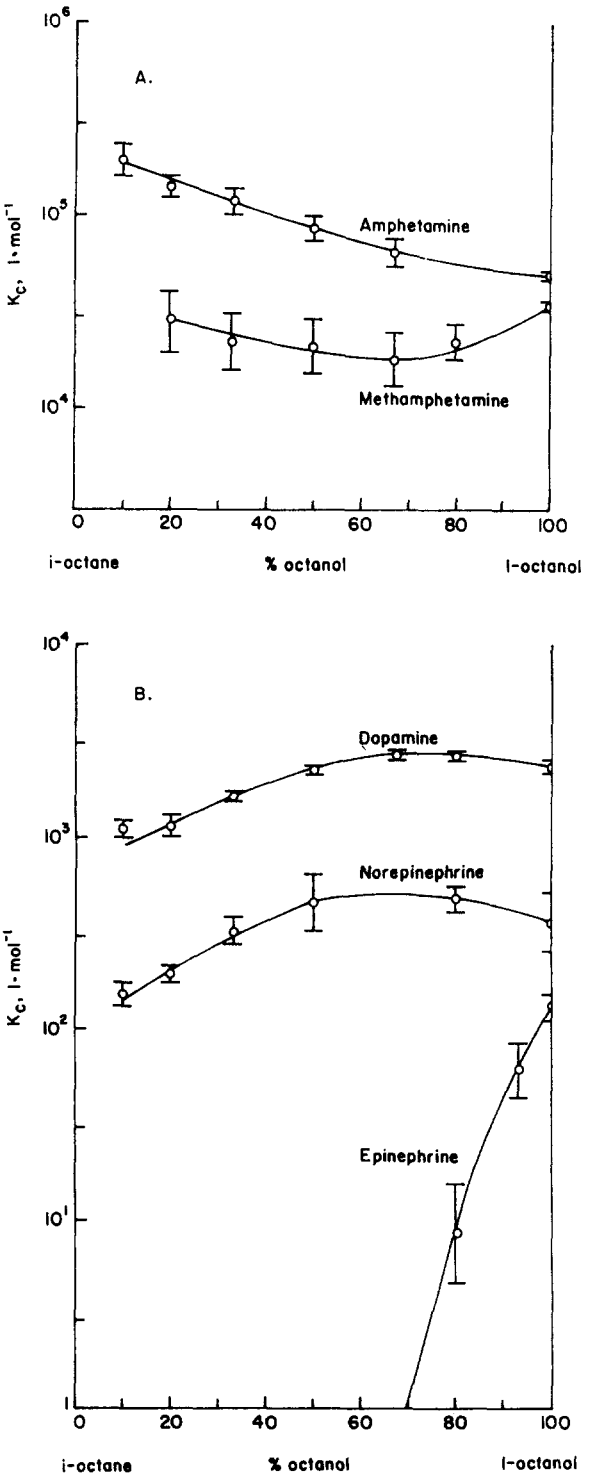

Fig. 2. Complex formation constants, K_c (l·mol^{-1}), for the ionophore-amine complex at 24°C plotted as a function of organic phase composition.

between ionophore and amine, leading to a lower K_C. The catecholamines (dopamine, norepinephrine and epinephrine, Fig. 2B) show an increase in K_C as the fraction of octanol in the organic phase approaches one. This is particularly dramatic in the case of epinephrine. When the ionophore-amine complex possesses exposed hydroxyl groups, as suggested by space filling models, octanol may better solublize these complexes through hydrogen bonding as reflected by an increase in K_C.

Bilayer membrane transport

Unidirectional amine flux across an X-537A doped membrane was measured, by two independent methods: [^{3}H]amine tracer monitoring and fluorimetric determination of the penetrating amine. All amines permeate the dioleoyl phosphatidylcholine/cholesterol bilayer in the presence, but not in the absence of X-537A. Plots of the amount of amine transported vs. time were linear for all amines studied (using either method of detection) differing only in slope as indicated in Table II.

The measured flux, J_{obs}, and flux estimated from conductance experiments, J_{est}, were compared for several amines under identical conditions (Table II). J_{est} was calculated from conductance experiments (ionophore concentration, $C_{X\text{-}537A} = 3.5$ mM, amine concentration, $C_{amine} = 50.0$ mM) using the Nernst Planck flux equation:

$$J_{est} = \frac{g_0}{Z^2F^2/RT}$$

where g_0 represents the membrane conductance, Z is the valence of the species which gives rise to the conductance (assumed to be ± 1), F is the Faraday constant, and R and T have their usual meanings. J_{obs} was calculated from the rate of appearance of amine across an X-537A doped membrane according to the equation:

$$J_{obs} = \frac{M_r}{t_{sec} \cdot A_m}$$

where M_r = the number of moles of amine in the receptor compartment and A_m = the area of the membrane in cm^2. J_{obs} may be obtained either from the slope of plots of $(cpm)_r$ vs. time (where

TABLE II

AMINE TRANSPORT ACROSS X-537A DOPED LIPID BILAYERS [a]

Amine	g_o [b] (mho·cm^{-2})	J_{est} [c] (mol·cm^{-2}·s^{-1})	J_{obs} [d] (mol·cm^{-2}·s^{-1})
p-Tyramine	$(5.0 \pm 0.9) \cdot 10^{-8}$ [e]	$1.3 \cdot 10^{-14}$	$(1.1 \pm 0.2) \cdot 10^{-8}$ [f,g,h]
m-Tyramine	$(1.7 \pm 0.7) \cdot 10^{-7}$ [e]	$4.5 \cdot 10^{-14}$	
β-Phenylethylamine	$(4.3 \pm 0.6) \cdot 10^{-8}$ [e]	$1.1 \cdot 10^{-14}$	$(7.0 \pm 0.3) \cdot 10^{-9}$ [f]
Amphetamine	$(2.2 \pm 0.2) \cdot 10^{-6}$ [e]	$5.9 \cdot 10^{-13}$	$(6.3 \pm 0.3) \cdot 10^{-9}$ [f]
Methamphetamine	$(1.2 \pm 0.2) \cdot 10^{-5}$	$3.2 \cdot 10^{-12}$	$(4.4 \pm 0.2) \cdot 10^{-9}$ [f]
Dopamine	$(5.4 \pm 0.6) \cdot 10^{-5}$ [e]	$1.4 \cdot 10^{-11}$	$(6.0 \pm 0.3) \cdot 10^{-8}$ [f]
Phenylephrine	$(4.8 \pm 0.3) \cdot 10^{-5}$ [e]	$1.3 \cdot 10^{-11}$	$(4.2 \pm 0.3) \cdot 10^{-9}$ [f,g]
Metanephrine	$(3.0 \pm 0.2) \cdot 10^{-6}$ [e]	$8.0 \cdot 10^{-13}$	$(4.5 \pm 0.4) \cdot 10^{-9}$ [f,g]
Norepinephrine	$(1.7 \pm 0.4) \cdot 10^{-5}$ [e]	$4.5 \cdot 10^{-12}$	$(2.0 \pm 0.5) \cdot 10^{-9}$ [g]
Epinephrine [i]	$(2.0 \pm 0.2) \cdot 10^{-6}$ [e]	$5.3 \cdot 10^{-13}$	$(7.0 \pm 0.6) \cdot 10^{-10}$ [g]

[a] Ionophore concentration in the membrane-forming lipid solution was 3.5 mM, amine concentration was 50.0 mM in pH 7.0 aqueous buffer solution.

[b] Specific membrane conductance, g_o; average $\pm$S.D. for four to six membranes.

[c] Flux estimated from the average specific membrane conductance, g_o, by the equation $J_{est} = g_o\, RT/F^2$.

[d] Observed unidirectional flux, J_{obs}, calculated as described in Materials and Methods; average $\pm$S.D. for two or three experiments.

[e] Specific membrane conductance from Ref. 11.

[f] Flux measured using [^{3}H]amine tracer, with unlabeled amine present on both sides of the membrane.

[g] Flux measured by a fluorescence technique, normalized for 50.0 mM amine; 10.0 mM amine was added to only one side of the membrane.

[h] *p*-Tyramine concentration for flux measurements was 10.0 mM.

[i] Epinephrine concentration for all measurements was 10.0 mM.

$(cpm)_r$ is the counts in the receptor compartment and is proportional to M_r) or from the slope of plots of amine concentration in the receptor compartment vs. time, normalized for membrane area (where amine concentration is determined fluorimetrically).

In all cases J_{obs}, which represents the total amine flux, is three to six order of magnitudes greater than J_{est}, a measure of the flux of charged (i.e., not 1:1 neutral) species, indicating that greater than 99% of the amine transport occurs as a 1:1 neutral or 'electrically silent' ionophore-amine complex. Amine permeability was independent of applied potential across the membrane further suggesting that the transported species is not charged. Comparison of absolute differences in magnitude of J_{obs} (determined from flux measurements) and J_{est} (estimated from specific membrane conductance determinations) may be questioned in light of Holz's observation [7] of the accelerative effect in transport of radiolabeled amine created by adding nonradioactive amine to the *trans* side of the membrane which then enhances the rate of return of the carrier to the *cis* side. However, the magnitude of the difference in flux determinations (a factor of 10^5) cannot be rationalized in terms of the modest concentration differences used in these two types of measurements. Furthermore, J_{obs} determined with radiolabeled amine and in its absence (by fluorescence techniques) yielded similar values for J_{obs} (Table II) substantiating the minimal influence of this '*trans*' effect in the interpretation of the data.

For transport across a membrane, the unidirectional flux of a species is proportional to its concentration gradient across the membrane, ΔC;

$$J = P_T \cdot \Delta C$$

where P_T is the total permeability allowed by the unstirred aqueous and membrane layers, P_{aq} and P_{mem}. P_{aq} and P_{mem} are defined by the following equations:

$$P_{aq} = \frac{D_{aq}}{h_{aq}}$$

$$P_{mem} = \frac{D_{mem} \cdot P_c}{h_{mem}}$$

where D_{aq} is the diffusion coefficient of amine through the unstirred aqueous layer, D_{mem} is the diffusion coefficient of the ionophore-amine complex through the membrane, h_{aq} and h_{mem} are the thickness of the two layers, and P_c is the membrane/water partition coefficient of amine.

Since all the amines studies were of similar molecular weight and structure, D_{aq} should not vary significantly for the different amines; h_{aq} and h_{mem} were experimentally controlled by regulating the rate of stirring and the lipid system chosen to form the bilayer. These experiments did not lend themselves to an accurate determination of the unstirred layer permeability. Membrane permeability of the amine was thus determined by the membrane/water partition coefficient of the amine, which takes into account the heterogeneous complexation reaction at the interface, and by the diffusion coefficient of the complex in the membrane. Thus, P_c may be governed by the magnitude of the ionophore-amine binding constant and the hydrophobicity of the amine-ionophore complex, whereas D_{mem} appears to be primarily dependent on the size of the complex and the viscosity of the bilayer.

As noted earlier, complexation constants measured by oil/water partition experiments also reflect the strength and hydrophobicity of the complex formed. Thus, the aqueous/organic partitioning of amine in the presence of ionophore and the amine flux across X-537A doped bilayer membrane may both serve as indicators of ionophore selectivity in transporting amines across membranes.

By comparing the permeability coefficients for several amines (Table I) the following approximate order of X-537A selectivity in their transport across bilayer membranes was determined: tyramine > β-phenylethylamine ~ amphetamine > dopamine > metanephrine ~ phenylephrine > norepinephrine > epinephrine. This order is consistent (within experimental error) with that determined independently by partition studies (Table I) and is in general agreement with preliminary monolayer measurements (surface potential-area isotherms) in which ionophore-amine interactions were studied at air/water interfaces to model the complexation at a membrane surface in the absence of complicating lipid-protein systems. The surface poten-

tial of the membrane increased as a function of the quantity of amine added to the aqueous phase (under the monolayer) allowing calculation of binding constants, which describe the ability of the ionophore to orient itself favorably at the interface, presumably a prerequisite for membrane transport. These determinations suggest that X-537A has affinity ranking for the above mentioned amines consistent with partitioning and lipid bilayer measurements. From partition measurements and flux studies, the affinity of the ionophore for methamphetamine appears to be less than for amphetamine.

In developing a structure-reactivity correlation, two factors need to be considered when evaluating X-537A selectivity for complexation and transport of substituted β-phenylethylamines:

(1) The number of hydroxyl groups on the amine. If the ionophore must desolvate the amine as a prerequisite for complexation at the membrane/aqueous interface, then increasing the number of hydroxyl groups (and the degree of solvation by the aqueous solvent) will decrease the amine's affinity for the ionophore.

(2) The number and position of methyl substituents. *N*-Methyl groups sterically hinder the approach of the ionophore to the amine nitrogen tending to decrease the magnitude of electrostatic interaction between them; whereas methyl groups which are not directly attached to nitrogen play little role in ordering selectivity.

In conclusion, a correlation can be drawn between the structure of substituted β-phenylethylamines and their propensity to complex with and be transported by by the ionophore X-537A. Furthermore, this selectivity determined from transport studies across bilayer membranes is consistent with partitioning and monolayer experiments.

Acknowledgments

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