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Adsorption of Aminopyridines to Phosphatidylserine Vesicles

Colin Wright
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AN ABSTRACT OF THE THESIS OF Colin Wright for the Master of Science in Physics presented May 22, 1986.

Title: Adsorption of Aminopyridines to Phosphatidylserine Vesicles.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:


Pavel Smejtek, Chairman


Jonathan J. Abramson


Makoto Takeo


David I. Clark

Intrinsic association constants were determined for a series of aminopyridines on phosphatidylserine vesicles, through determination of electrophoretic mobility. The magnitudes of the constants were such that depolarization of the nerve terminal through binding to negative phospholipids seems unlikely to occur. The aminopyridines all had association constants between one half and one

sixtieth the association constant of calcium. The aminopyridines probably block potassium channels in their enhancement of synaptic transmission.

An interesting correlation was noted in that the ranking of the aminopyridines in terms of their affinity for these lipids was approximately the same as the ranking of their enhancement of synaptic transmission in a wide variety of biological preparations. A possible mechanism for this correlation is that adsorption of these drugs to lipid membranes is the initial and rate-limiting step in their action.

ADSORPTION OF AMINOPYRIDINES TO
PHOSPHATIDYLSERINE VESICLES

by

COLIN WRIGHT

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
PHYSICS

Portland State University

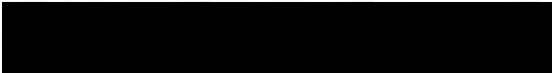
1986

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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CHAPTER I

INTRODUCTION

This thesis describes a study of the adsorption of a series of aminopyridines to phosphatidylserine multilamellar vesicles. Phosphatidylserine is one of the major negatively charged lipids that have been found in cell membranes. Multilamellar vesicles are the thermodynamically stable form of a suspension of these lipids in water. The rationale behind the study is to gain insight into the biological mode of action of the aminopyridines. They have been found to facilitate synaptic transmission, that is the jumping of a neural impulse from one neuron to the next.

The underlying problem is essentially this: how does the incoming action potential liberate the neurotransmitter, the chemical substance that carries the signal across the synaptic cleft? (The synaptic cleft is the small region (40-100 angstroms) between the end of one nerve cell and the start of another. See Figure 1.)

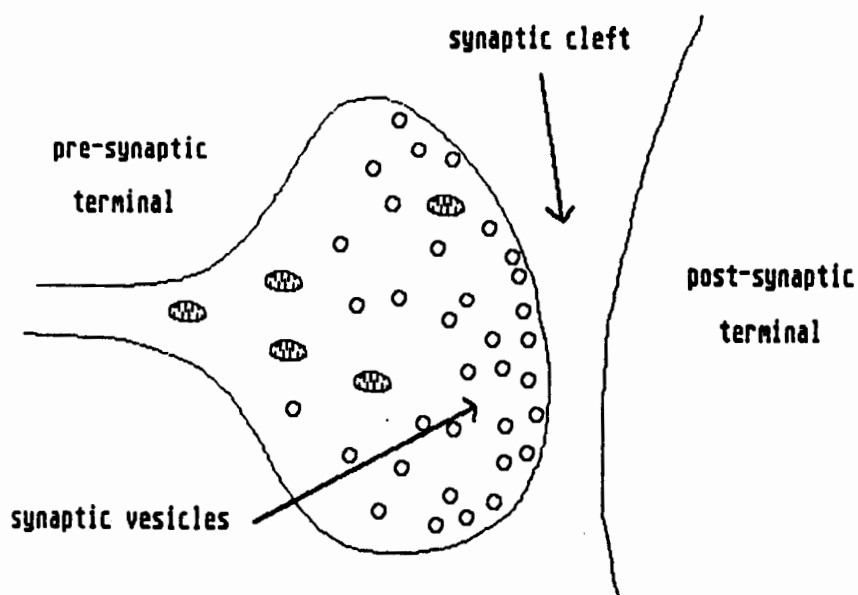


Figure 1. A representative synapse

What is known about this transmission process is that inside the presynaptic nerve terminal are minute synaptic vesicles, each about 250 Å in radius, and filled with several thousand neurotransmitter molecules (1). The inside of the neuron is held at about -60 to -70 millivolts with respect to the outside of the neuron. When the incoming signal, the propagating action potential, "depolarizes" (i.e. makes more positive) the inside of the nerve terminal, the vesicles adjacent to the cell membrane fuse with it, liberating the transmitter into the synaptic cleft. From here, the transmitter molecules diffuse to the postsynaptic membrane where they activate a response.

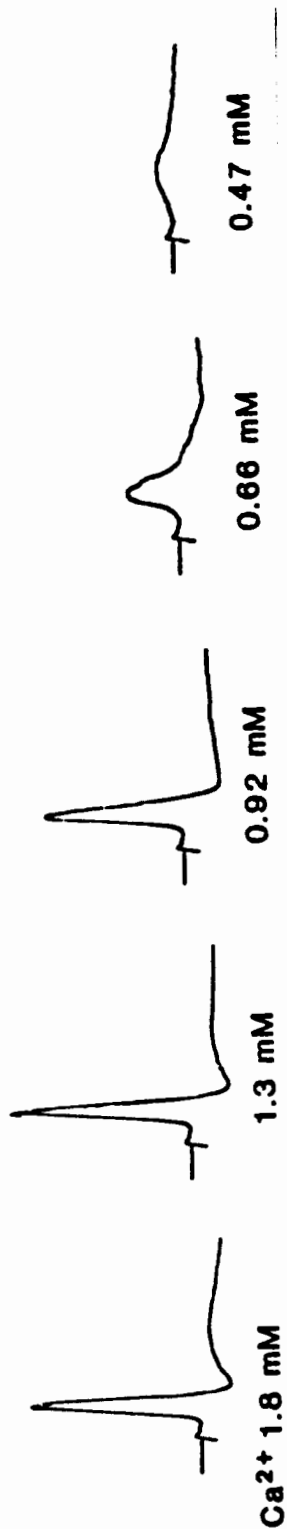
Katz and Miledi suggested correctly that intracellular calcium is the determinant for transmitter release (2). When the action potential arrives, the permeability of the

membrane to calcium increases rapidly, calcium flows into the neuron and the vesicles are released. If no calcium is available in the external solution, no vesicles are released when the membrane is depolarized.

Various aminopyridines have been found to enhance this synaptic transmission process (e.g. see reference 3), i.e. higher postsynaptic potentials are generated. They have also been found to be able to substitute for a certain proportion of the extra-cellular calcium. That is, a lower concentration of external calcium can be compensated for by a small amount of aminopyridine, to give the same postsynaptic response. Figure 2 presents these ideas in the form of experimental data collected by Matsomoto and Riker from the Oregon Health Sciences University. The traces represent the average postsynaptic response as the external calcium concentration is reduced as indicated. In the top set, we see almost complete transmission failure at about 0.5 millimolar calcium, whereas, in the bottom set, with the addition of 0.1 millimolar of an aminopyridine (in this case, 3,4-diaminopyridine) transmission failure occurs at the much lower level of 0.1 mM calcium.

This might suggest a competition between the aminopyridine and the calcium for a common site or domain on the presynaptic external membrane terminal (4). On the other hand, some studies suggest that the aminopyridines act intracellularly (5), where the calcium concentration is

Drug-Free



3,4-DAP (100 μ M)

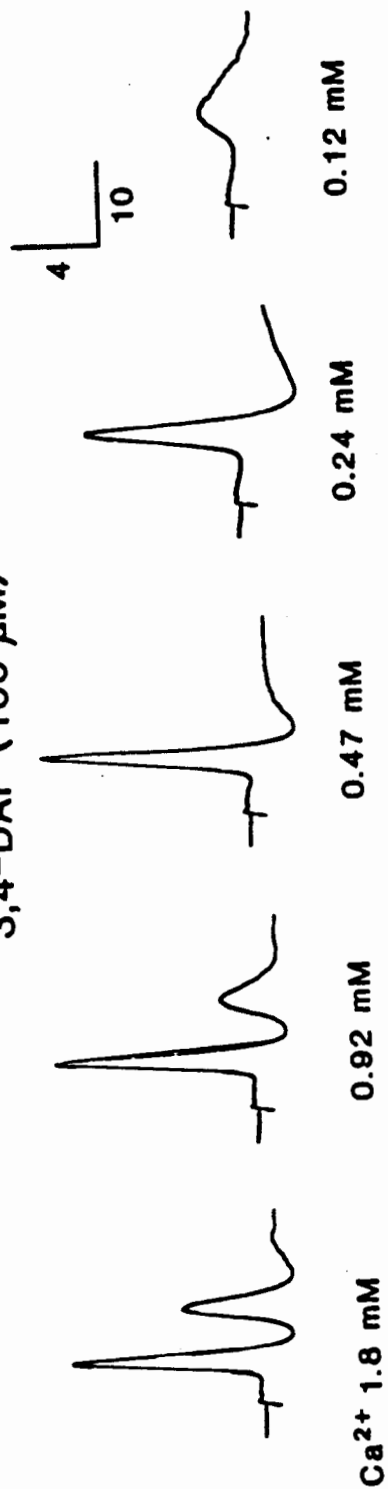


Figure 2. Preservation of synaptic transmission by 3,4-diaminopyridine in low calcium solution. Traces are the extracellular recordings of postganglionic responses to preganglionic stimulation in the same isolated bullfrog sympathetic ganglion. From Matsumoto and Riker (3).

kept at very low concentrations by active mechanisms of the cell. By interfering with potassium channels, the aminopyridines may be altering the action potential with the result that more calcium enters the cell.

To test the former possibility, that of a competition for sites on the presynaptic membrane, a study of the binding of calcium and aminopyridines to a representative lipid was undertaken. Phenomenological association constants were determined for each of 7 aminopyridines plus 1 analog. (See Figures 3 and 4 for molecular structures and abbreviations used.) The magnitudes of these constants were fairly low, but their order correlated remarkably well with the biological potency of this series of aminopyridines. A proposed mechanism is given to explain this correlation in the conclusion of the thesis. The main result too is announced there, that it does not appear likely that aminopyridines can bind sufficiently strongly to lipids to account for their remarkable properties.

This chapter concludes with a brief and slightly technical resume of the history of aminopyridines in medical science in an attempt to put in perspective the problem we are dealing with. Chapter two describes both the computational and the experimental aspects of the determination of the association constants. With regard to the former, a short, non-rigorous introduction to

electrical double layer theory is given in Appendix A. This forms an essential part in accounting for the adsorption of a charged species from solution onto a surface (in our case, lipid vesicles). Chapter three begins with a summary of the experimental results (the individual data are relegated to Appendix B), and is followed by a discussion. Finally, a conclusion is offered in chapter five.

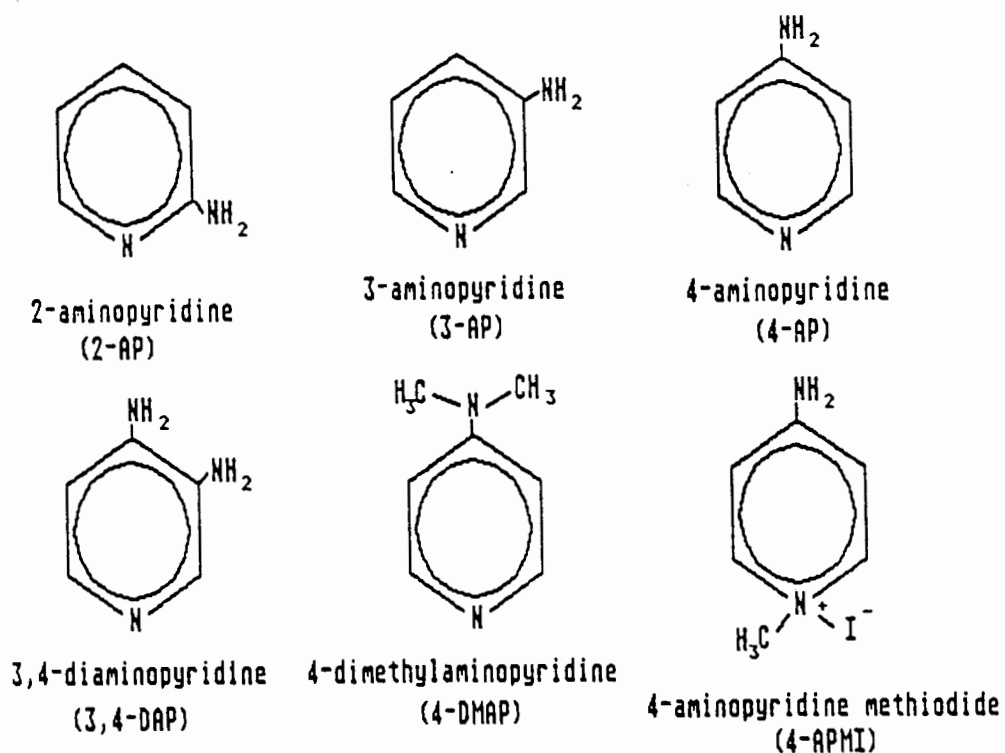


Figure 3. Aminopyridines studied and their abbreviations

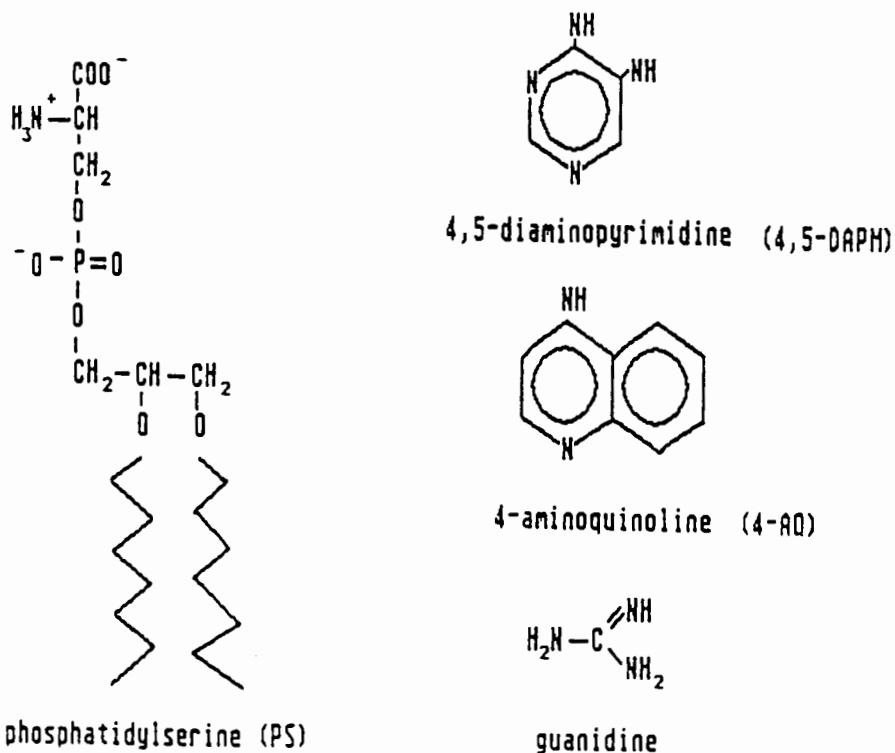


Figure 4. Phosphatidylserine and other molecules mentioned in the text.

AMINOPYRIDINES

Many of the pharmacological properties of aminopyridines, including their vasopressor and convulsant actions, were described by Dohrn in 1924 (6). However, over 30 years elapsed until there were further studies on the action of aminopyridine on nerve-muscle preparations. These studies clarified that aminopyridines facilitate transmitter release, not only at the neuromuscular junction but also at a wide range of synapses. Work, particularly in France, culminated in the discovery that aminopyridines were selective blockers of potassium channels (Pelhate et

al (1974) (7)). This provided an explanation for some of the pharmacological actions of the drugs and, furthermore provided researchers with a useful probe of excitable membranes. In this latter respect, aminopyridines offered a number of advantages over tetraethylammonium (TEA), the classical inhibitor of potassium conductance. Today, interest in these compounds has increased enough to warrant an international conference devoted to their actions. (The conference, which was held in 1981 had over 100 participants from 25 different countries (8)).

Extensive reviews exist that summarize the various studies that have been performed on many different biological preparations with aminopyridines (9, 10) (e.g. see Glover (9) with over 200 references). In particular, it is now clearly established that aminopyridines block potassium channels in a wide range of excitable membranes (frog sciatic nerve, cat hind paw, cockroach axon, squid giant axon, etc), irrespective of the type of transmitter substance (e.g. acetylcholine, noradrenaline, etc.). They are generally more potent than TEA and have different blocking kinetics (11,12). The aminopyridines also have valuable potential clinical applications. They have been used as an antagonist to muscle paralysis in botulism and various muscular diseases, such as Eaton-Lambert syndrome (13).

Questions remain concerning specific effects of the

aminopyridines. For instance, in squid, 4-AP causes depolarization and induces repetitive firing (14). In some experiments, in high concentrations, they were found to increase spontaneous (that is, in the absence of direct stimulation) transmitter release (15), and, in others, to produce repetitive post-synaptic potentials, in response to single shock stimulation (in frog (16)). Thus, in some preparations, they increase membrane excitability. In addition, in frog sartorius, low doses of 4-AP enhance twitching without modifying the action potential (17), while in mouse diaphragm 4-AP prolongs action potential without modifying muscle contractility (18). Finally, Riker and Matsumoto noticed no broadening of action potentials in the individual nerve cells that contributed to the averaged traces of Figure 2 (3).

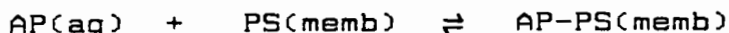
These kinds of studies have led certain researchers to propose new modes of action for the aminopyridines. For our purposes we can quote Kim et al. (19), who suggested that there may be two possible ways in which quaternary ammonium compounds could promote entry of calcium into the nerve terminal: " (1) through increased duration of the action potential, and (2) by reduction in the electrostatic screening of fixed negative charges on the nerve membrane." This thesis could be considered as arising to test this latter possibility.

CHAPTER II

In this chapter, we begin by defining the binding constants that we are setting out to measure. The means of measurement will be through determination of electrophoretic mobility. Consequently, we give a description of the Helmholtz-Smoluchowski equation which is necessary to relate the mobility to an electrical parameter, the electrokinetic or zeta potential. From Appendix A, which contains a short introduction to the Gouy-Chapman theory of the electrical double layer, we are then able to calculate surface charge densities from the zeta potentials. Finally, a Langmuir adsorption isotherm is described from which we can obtain the binding constants from the surface charges. Details of the computation are followed by a section on the experimental methods.

DEFINITION OF BINDING CONSTANT

To quantify the postulated binding, we use the equilibrium binding constants defined by the one-to-one reaction,



representing the adsorption of the aminopyridine onto the phosphatidylserine membrane. Here AP-PS represents the bound aminopyridine phosphatidylserine complex. Thus, we have, for the equilibrium binding constant, K,

$$K = \frac{(\text{AP-PS})}{[\text{AP}]_0 (\text{PS})} \quad (1)$$

where the parentheses represent surface concentrations on the membrane and the square brackets aqueous concentrations. A source of ambiguity arises here, in that part of the binding is electrostatic in origin--the membrane will be negatively-charged, the aminopyridines univalent cations. Because the electrostatic contribution will depend on the state of charge of the membrane, we define K to be the "intrinsic" binding constant by correcting for the electrostatic attraction with a Boltzmann factor applied to the aminopyridine bulk concentration. That is,

$$[\text{AP}]_0 = [\text{AP}]_b \exp(-e U_0/k_b T)$$

where $[\text{AP}]_0$ is the concentration adjacent to the surface (where the potential is U_0 and will be negative), $[\text{AP}]_b$ is the concentration in the bulk solution (where the potential will be zero), and e , k_b , and T are the proton charge, Boltzmann constant and absolute temperature respectively.

To measure K we will need some means of measuring the surface concentration of adsorbed aminopyridine on phosphatidylserine membranes. To this end, we are aided by the fact that phosphatidylserine forms small spherical vesicles--ready-made membrane surfaces, composed of bimolecular layers--when shaken in an aqueous medium. The vesicles are visible under a microscope, and since they are negatively charged, they will drift in an applied electric field. Furthermore, since the aminopyridines are univalent cations, on adsorption they will tend to neutralize the lipid vesicles. The stronger the adsorption process (the higher the value of K), the more aminopyridine and positive charge on the vesicle surface at any one time and hence the slower the vesicle will move. Thus we have a way of comparing the actions of a variety of aminopyridines. A stopwatch and a graduated eyepiece will provide us with the raw data, i.e. the drift velocities, necessary to quantify the amount of binding. With an appropriate model we are then able to calculate the equilibrium binding constants we seek.

Already, in our above discussion of the Boltzmann factor, we have come into contact with the Gouy-Chapman theory of the electrical double layer--the attraction of mobile ions to a charged surface. This might be a good time to refer the interested reader to Appendix A, where a brief description of the theory and the mathematical

equations that we will be using later are developed. Important for the next section is the notion of the "thickness" of the diffuse layer, the Debye screening length.

THE HELMHOLTZ-SMOLUCHOWSKI EQUATION

In general, there is a very complicated relationship between the charge on a particle and the drift velocity it will attain in an applied electric field. If we were dealing with, say, a charged bubble falling through an electric field, then our analysis would be simple. Here the Stokes' resistance (for low Reynolds number) balances the electrical force,

$$6 \pi \eta a v = Q E$$

or,

$$\mu = v/E = Q / (6 \pi \eta a)$$

where μ defines the mobility, η is the viscosity of the medium and a the particle radius.

However, in our case, the above relationship is invalid because we would be neglecting the effects of the diffuse cloud of oppositely-charged ions that will tend to collect around the particle. (Even in pure, distilled water there will be ions due to the dissociation of the water molecules.) The cloud will tend to move in the opposite direction to the particle in an applied electric

field, and thus the particle will effectively have to move "upstream". This is called the electrophoretic retardation effect. Furthermore, the "center of gravity" of the charged cloud will tend to lag behind the "center of gravity" of the particle, thus creating an additional force. This is sometimes referred to as the retardation effect. Because of these complications, exact solutions to the hydrodynamic equations of motion are few, and then only under restrictive assumptions. The reader is referred to Overbeek and Wiersema's article for more detailed discussion (20).

Fortunately, in the case when the "thickness" (Debye length) of the diffuse cloud is small in comparison to the radius of the particle, simplifications occur. First of all, we can approximate the natural spherical geometry of the particle locally as a flat surface and proceed to make use of the results of Gouy-Chapman theory. Secondly, we can neglect the retardation effect in the calculation of the particle's motion.

Furthermore, we can arrange to reduce the "thickness" of the diffuse double layer, simply by adding more electrolyte to the solution. In our case, the vesicles are approximately 1-10 micrometers in diameter and, with 0.1 M background electrolyte, the double layer "thickness" will be less than 10 angstroms. Thus, we have a ratio of particle size to double-layer "thickness" of

more than one thousand to one, and the flat geometry will introduce only very small errors.

Armed then with our thin double layer, we can proceed to obtain a simple relationship between the particle mobility and its charge, first obtained by Helmholtz in 1879 and Smoluchowski in 1903. Actually the electric potential at the particle surface will turn out to be a more convenient variable than the particle charge, although here we must be careful by what we mean by "particle surface." It is a long accepted part of classical hydrodynamics that the fluid velocity at the surface of a rigid particle in a viscous medium is zero. Here we mean both the normal component of velocity and also the tangential component (the latter stipulation being the so-called non-slip hypothesis.) Thus, at least the layer of water immediately adjacent to the particle will stick to it, and the radius of our particle will have increased.

The electric potential at this "surface of shear", is the definition of the electrokinetic or zeta potential (ζ). The zeta potential is what is measured in these experiments, not the surface potential from which it can have differ substantially. Assumptions must be made to relate these two potentials. In concordance with other workers in the field (21), we take the distance to the plane of shear to be 2 angstroms. (We note, however, that this distance is a function of ionic strength (or possibly,

electric field strength (22)), and that Davies and Rideal treat the case of a diffuse rather than a sharp plane of shear (23).)

Continuing with the derivation, then, with the flat geometry, and x representing the distance normal to the charged surface, the external force exerted on a layer of liquid of thickness dx is $\rho E dx$, with ρ the space charge density (which moves with the particle) and E the electric field strength. This force will be purely balanced by the viscous force on the same shell (no pressure gradient need be invoked to satisfy the equations of motion), so that

$$\eta d^2v/dx^2 = \rho E$$

with η the bulk viscosity and v the fluid velocity. However, since the space charge density is given by the Poisson equation, we have (in MKS units)

$$\eta d^2v/dx^2 = (-\epsilon d^2\psi/dx^2) E$$

($\epsilon = \epsilon_0 \epsilon_r$ is the product of the permittivity of free space and the dielectric constant and ψ is the electric potential.) Two integrations and the use of the boundary conditions ψ , $d\psi/dx$, v , and dv/dx all equal to zero at large distances from the particle leave us with

$$v = -(\epsilon\psi/\eta) E .$$

The action of the applied field will be to impart a

velocity, U , to the particle. On a coordinate system fixed to the particle, the fluid velocity at large distances will be $-U$ and we then have

$$\mu = U/E = \epsilon \zeta / \eta ,$$

the Helmholtz-Smoluchowski equation relating the mobility of our charged particle, with ζ , the zeta potential, a result independent of the particle size.

For completeness, we mention that in the case when the size of the particle is much smaller than the diffuse double layer thickness, the value for the mobility is two thirds as much and can be shown (24) to reduce to $Q/6\pi\eta a$, the Stokes' result mentioned at the start of this section. In the intermediate case, when neither particle size nor diffuse layer thickness dominate, there is no closed form solution, but the theory has been given by Henry and is included in Rice and Nagasawa's book (25), along with a good discussion of inertial and relaxation effects.

So far then, we have an expression for the potential at the plane of shear. Our next step will be to relate the zeta potential (via the surface potential) to the surface charge on the particle and thence to the amount of bound aminopyridine. With an appropriate adsorption model, we can then extract the binding constants we are looking for.

DESCRIPTION OF ADSORPTION MODEL

In surface science the relation between the amount of adsorbed material and the amount in solution is called the adsorption isotherm. One of the simplest of these is the Langmuir adsorption isotherm, developed in 1917, which provides a fixed lattice onto which the adsorbate binds.

By simple kinetic arguments, the fraction of bound sites, θ , will be proportional to the free ionic concentration at the membrane surface, in our case $[AP]_0$, (i.e. the bulk concentration times a Boltzmann factor). θ will also be proportional to the fraction of free sites available to bind, $(1 - \theta)$. The intrinsic binding constant, K , is then the proportionality constant,

$$\theta = K [AP]_0 (1 - \theta) .$$

This can be seen to be equivalent to our previous definition of K , equation (1) above, by dividing this latter equation through by the total number of binding sites.

It is easy to see that the surface charge density, σ , will decrease in proportion to θ :

$$\sigma = \sigma_0 (1 - \theta)$$

where σ_0 is the maximum charge density, i.e. before any adsorbate has been introduced. Solving between these two equations we find

$$\sigma = \sigma_0 / (1 + K [AP]_0) \quad (2)$$

This equation provides the link with double layer theory, in that σ_0 is related to the surface potential through the Grahame equation (A1) in Appendix A, and to the experimental zeta potentials through the potential profile equation (A2), also in Appendix A. K and σ_0 , then, are our unknowns. From plots of zeta potential against aqueous concentration we can extract these constants.

COMPUTATIONAL METHODS

Initially, attempts were made to extract the binding parameters, K and σ_0 , directly from linear plots of the isotherm raw data. However, due to scatter in the data, consistent values could not be obtained and another approach was adopted. This was to minimize the chi-square deviation of the experimental data from the modeled theoretical data, assuming σ_0 to be 1 negative charge per 65 square angstroms, a value in agreement with others in the field (21). The chi-square deviation, χ^2 , is the sum of the squared differences between experimental and theoretical zeta potentials, each point weighed according to its own experimental standard deviation squared, σ_i^2 ,

$$\chi^2 = \sum_i (\zeta_i^{\text{theor}} - \zeta_i^{\text{exp}})^2 / \sigma_i^2,$$

where the sum runs over the concentration points of a

particular isotherm, collected as described below.

If we now turn to Appendix B, we can see this more clearly. In the figures, experimental mobilities, converted to zeta potentials with the Helmholtz-Smoluchowski equation, are plotted as a function of aqueous aminopyridine concentration. The two drawn lines were theoretically computed using the Gouy-Chapman and Langmuir theories. The bottom darker curve is the screening or electrostatic contribution to binding, corresponding to $K = 0$. The line above indicates the "tight binding", over and above the screening. The value of K which reproduces this latter curve is indicated in the top left hand corner of each figure. It is this value which minimizes the chi-square deviation as defined above.

To implement the procedure, K was first assumed to be zero and the theoretical zeta potential was calculated via the Gouy-Chapman equations (A1) and (A2), using $\sigma = \sigma_0$, and $x = 2 \text{ \AA}$, the distance to the shear plane. The chi-square was then found. Then K was increased slightly and the new value of surface potential calculated by solving between equation (2) above and (A1) from Appendix A. (The potential in equation (2) enters through the Boltzman factor in $[AP]_0$.) There is only one value of V_0 that simultaneously satisfies both (2) and (A1). In our case, that value was found by balancing the surface charge density with the integral of the space charge density, i.e.

with a subroutine that found the required root of $(\sigma - \int \rho dx)$. (There are, of course, other ways of getting at the correct value for U_0). With U_0 known, equation (A2) gives us the zeta potential and the new chi-square value could be obtained.

Then K was increased further in small steps until the value which minimized the chi-square deviation of the experimental and theoretical zeta potentials was found. Care was taken to ensure that the value of K found did not correspond to a local minima. The calculations were performed on a PDP 11/23 minicomputer with DIUKA1, a Fortran program developed by Dr. Smejtek. The tables following the figures in Appendix B show the numerical values obtained. We see the agreement is quite good.

EXPERIMENTAL METHODS

Now that the approach to the problem is outlined, we can proceed to describe the experimental determination of particle mobility.

The disodium salt of bovine brain phosphatidylserine (20 mg/ml) was obtained from Avanti Polar Lipids (Birmingham, Alabama) and used without further treatment. A 0.5-1.0 ml aliquot of the lipid was mixed with about 50 ml of analytical grade chloroform. The chloroform was distilled off in a rotary-evaporator and collected in an ice-cooled receiver, while the residue lipid formed a thin

layer on the bottom of the round-bottomed flask.

After an hour of sitting in the vacuum of the evaporator to ensure that all the chloroform had been removed from the lipid, the flask was shaken with 15-30 ml of a buffered solution containing 0.1 M tetramethylammonium chloride and 1 mM EDTA to form a cloudy suspension of vesicles.

Tetramethylammonium chloride was the background electrolyte used, since it has a very low association constant with phosphatidylserine ($K < .05 \text{ M}^{-1}$ (21)) and thus would simply "screen" the charged surface and not bind to it. The buffer for the pH 7.2 runs was MOPS (3-[N-morpholino]propanesulfonic acid), while a general-purpose borate/citrate/phosphate buffer was used for the other pH's. The reason for this was that three of the compounds were only weakly ionized at neutral pH, and so their association constants had to be obtained at pH's were there ~~4-~~ was significant ionization. Values of 80 % and 20 % ionization were chosen, (using pKa values of 6.03, 5.98, 6.86 for 4,5-DAPM, 3-AP and 2-AP respectively), and the averaged value for K was used.

A 1 molar stock solution of each aminopyridine in buffer was made up, from which 10X and 100X dilutions were made. The source of 4-AP, 3-AP and 3,4-DAP was Aldrich Chemical Company Inc. (Milwaukee, Wisconsin). 4,5-DAPM and 4-DMAP were from Sigma Chemical Company (St. Louis,

Missouri), while 2-AP was from Reilly Chemicals. We thank Dr. Riker for providing us with many of the aminopyridines.

For each of twelve concentrations, 1 ml of lipid stock together with an appropriate volume of aminopyridine were made up to a total volume of 10 mls with buffer. These then were used in the electrophoretic apparatus in order of increasing concentration.

The electrophoretic apparatus (Rank Brothers, Mark I, Bottisham, Cambridge, U.K.) consists of a U-shaped cylindrical cell (total volume 6 ml), a microscope ($\times 40$ objective), a d.c. power supply, two platinum electrodes and a water bath. The water bath was set to 25.0 ± 0.5 degrees centigrade for all measurements. An applied d.c. voltage of 20-100 V caused the vesicles to drift at measurable velocities. Voltage and current were monitored with a Data Precision 245 multimeter and a Dana Multimeter 4200, respectively.

In the steady state, applied voltages tended to drop slowly due to electrode polarization, i.e. small back e.m.f.'s were generated. To minimize this effect, and to average out any slight undesired drift velocities that might be occurring in the bulk solution, the applied voltage was alternately reversed in sign and the timing repeated, with the particles moving in the opposite direction. (The two velocities obtained were averaged in the data analysis: they usually agreed to within a couple of percent.) In any

case, polarization was never allowed to exceed about 2% (i.e. no more than a 0.5 V voltage drop over a 20.0 V applied e.m.f.).

Particle velocity was obtained as a function of depth of microscope focus. The reason for this is that there will be a contribution to the particle velocity due to the movement of bulk liquid that the ions will carry along with them in the applied field. The bulk liquid velocity has a parabolic profile, but since the cell is closed there can be no net movement of water. It is not difficult to show with these two pieces of information that at a radius of $R / \sqrt{2}$ where R is the radius of the cylinder, the bulk velocity has to be zero. This radius corresponds to the so-called stationary layer, and the vesicles will be moving solely only under their own charge at this position.

Generally at least 20 measurements were made of velocity for each concentration point. The raw data was analysed on a PDP 11 minicomputer, using ELPH4, a Fortran program developed previously in the Membrane Biophysics lab to obtain the linear least squares fit for the velocity at the stationary layer--the layer in the electrophoretic cell where there is no bulk movement of liquid. The mobilities were all independent of particle size (as expected).

Control measurements on free vesicles in buffer solution indicated zeta potentials comparable to published data (21). There was no evidence to suggest that the

interlipid distance changed on adsorption of aminopyridine (which would consequently effect our adsorption model) (26). Probably, since the aminopyridines are ions they will reside on the membrane surface where we expect pockets of potential minima (27) to be, and so not appreciably alter the interlipid distance.

CHAPTER III

At the end of the first chapter, the rationale behind this study was stated. Namely, two hypotheses concerning the enhanced entry of calcium into nerve terminals in the presence of aminopyridine were mentioned. One hypothesis had it that the aminopyridines were able to bind to the membrane surface and reduce the screening, the other that they were blocking potassium channels and prolonging the action potentials. In this chapter, we develop the two hypotheses further and will see that the latter is the more tenable.

First, though, we summarize the work of chapter two and notice a remarkable correlation between the binding constants and the biological activity of the aminopyridines.

RESULTS AND DISCUSSION

Results, in descending order of binding efficiency, are presented in Table 1. These represent the core of the thesis. The association constant of calcium (assuming 1:1 binding to PS), is also included. We notice all the

aminopyridines bind more weakly than calcium.

Membrane active ion	Association constant (M^{-1})	Standard deviation
calcium	12.1	1.3
3,4-diaminopyridine	6.5	1.0
4,5-diaminopyrimidine (*)	3.8	1.3
4-aminopyridine	2.6	0.5
3-aminopyridine (*)	1.8	1.0
2-aminopyridine (*)	1.6	0.8
4-dimethylaminopyridine	0.5	0.3
4-aminopyridine methiodide	0.2	0.2

TABLE I

EXPERIMENTAL ASSOCIATION CONSTANTS (M^{-1})
(*) = AVERAGE AT TWO PH'S

An interesting piece of information comes to light when we look at some recent results obtained by Matsumoto and Riker (28, 29). They found the following sequence for enhancement of synaptic transmission, in the bullfrog sympathetic ganglia:



(See Figure 3, for formulas and abbreviations.) Aside from our analog species, 4-5-diaminopyrimidine, this order corresponds quite well with the order we have obtained above for the binding constants. (In fact, the order is exact, but for DMAP and 4PMI whom we notice fall within each other's standard deviation.)

Our sequence is also in agreement with Molgo et al. (5), who obtained the following ranking of enhanced evoked transmitter release,

3,4-DAP > 4-AP > 4-AQ > 3-AP > 2-AP

for the frog neuromuscular junction by measuring quantal content, in the absence and presence of the aminopyridines. (The quantal content is given by the ratio of mean end plate potential (EPP) to mean miniature end-plate potential (MEPP). The MEPP would be the change in postsynaptic potential per quantum (synaptic vesicle's worth) of neurotransmitter, while the EPP would be the cumulative postsynaptic potential generated.)

We conclude that the agreement between the order of biological potency and the binding constants to PS is very strong.

THE SURFACE POTENTIAL THEORY

In order to understand how aminopyridines could have facilitatory effects on the calcium dependence of synaptic transmission, it will be useful to review the basic theory of the action potential, and discuss the importance of calcium in the "surface potential theory".

With the advent of the voltage clamp technique (due primarily to Cole), which enabled the voltage-current characteristics of the nerve cell to be monitored,

Hodgkin and Huxley (30) were able to advance their phenomenological theory of nerve transmission (in 1952). In their theory, the transmembrane current is decomposed into two components, an inward transient sodium current and a steady, outward potassium flow. The driving forces for these currents are the respective gradients of electrochemical potentials set up by active (i.e. energy consuming) transport through the membrane, which leave the membrane interior at a resting potential of -60 to -70 mV with respect to the exterior.

Calcium (which has been known to be an essential requirement of neural transmission since the beginning of the century), when present in the external bathing solution, was soon found to simply shift the conductivity-voltage curves to the left along the voltage axis. The depolarization needed to reach firing threshold also increased. Calcium was thus found to have a stabilizing influence on the neuron.

A possible explanation for these results was advanced by Huxley in 1957 (31), who postulated the existence of a layer of fixed negative charge on the outside surface of the membrane. The consequent negative surface potential creates an electric field that adds to the external field set up by the ionic concentration gradients. It is this total field that is sensed by the membrane components that control the conductivity of the membrane. He further

postulated that cations should reduce the negative surface potential by binding to the surface. Hence calcium added to the external bathing solution should increase the depolarization needed to bring the field within the membrane to a given value large enough to open the sodium channels, i.e. they hyperpolarize the membrane. (See Figure 5, where adding cations would shift curve I to curve II).

In a refinement of the surface potential theory, McLaughlin, Szabo and Eisenman (32), in 1971, clarified that cations can be effective in producing these voltage shifts by purely screening the surface charges (and not just by simply binding). In a further application of the theory, McLaughlin and Harary (1974) (33) applied the Boltzmann relationship to the distribution of charged lipids in a lipid bilayer and suggested that if phospholipids can "flip-flop" to redistribute themselves between inner and outer leaflets then they can effectively reduce the external field. Thus, we can in general expect an asymmetrical distribution of charged lipids across a membrane.

This membrane asymmetry in surface charge, and consequently surface potential, is indicated in Figure 5, which is taken from Hille, Woodhull and Shapiro (1975) (34). In this paper, they give evidence for a high density of negative charge near sodium channels using the surface

potential hypothesis. With reference to the figure, we note that on depolarization, the potential inside the neuron increases dramatically in the positive direction. According to their estimates then of surface potentials, the transmembrane field will then change direction, and the possibility arises that channels (in our case, we are interested in the calcium channels), can be opened and closed by changes in the transmembrane electric field.

The importance of negative surface charges on membranes seems to be gaining more recognition. Proof that the surface charges are not restricted to the axon alone comes from researchers (e.g. (35)) who have obtained high negative mobilities for cerebral-cortex synaptic vesicles and synaptosomes--vesicle preparations of nerve terminals. There is nevertheless a slow acceptance of the surface potential theory in the field, perhaps because the surface potentials cannot be measured directly with electrodes--the charged region is neutralized within tens of angstroms from the surface, as we have seen. Nevertheless, the consequences of the theory, particularly with respect to the functioning of channels, may be important.

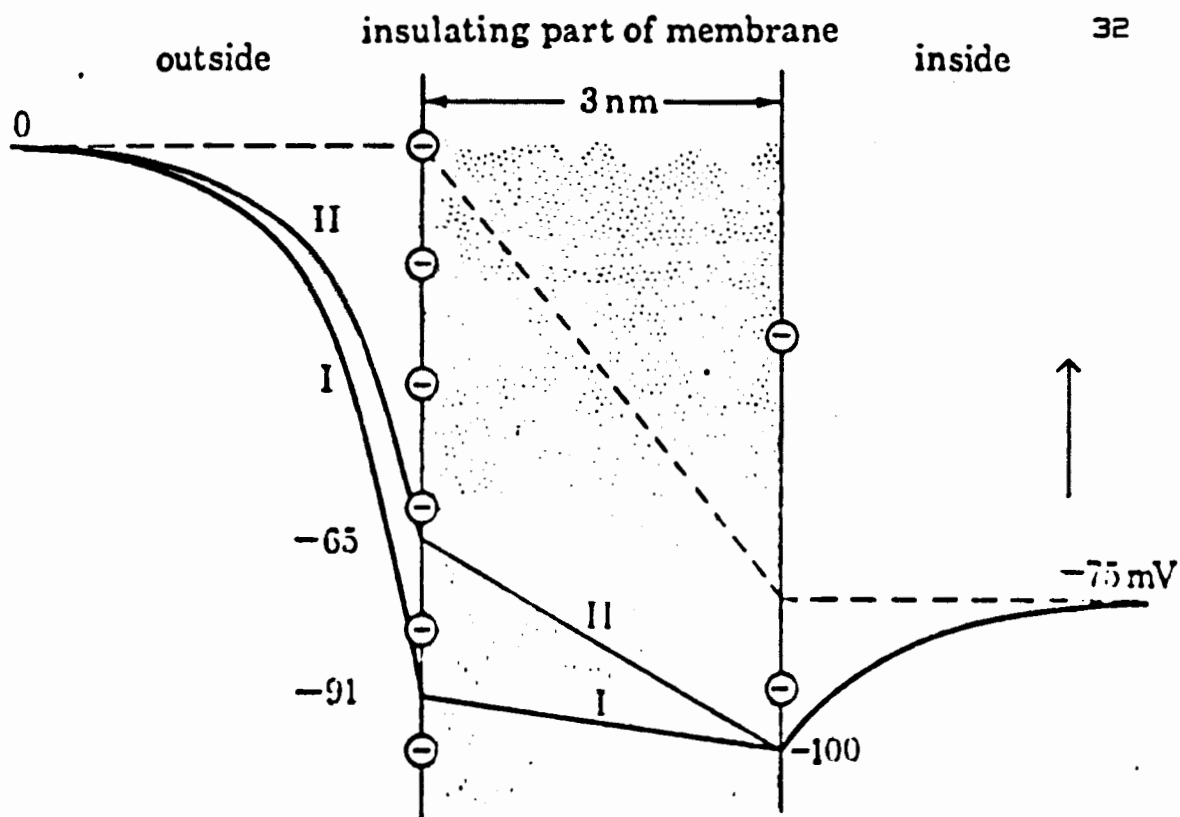


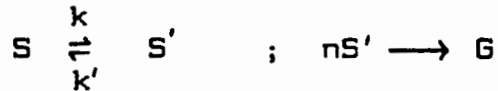
Figure 5. Postulated potential profile across an axon membrane (due to Hille et al. (34)). The arrow shows how the potential in the inner region changes on depolarization.

A MODEL OF THE CALCIUM CHANNEL

The voltage dependence of calcium channel functioning has been measured by Llinas and coworkers, who have also developed a mathematical model of a presynaptic calcium channel. The model gives a reasonable fit to their voltage clamp data of squid stellate ganglion (1, 36, 37). By blocking sodium and potassium currents, they were able to monitor calcium current under various levels of controlled presynaptic depolarization.

In their model, which is of the Hodgkin-Huxley type,

they begin with the assumption that each calcium channel is composed of n subunits. Furthermore, they use a two-state kinetic model to describe each subunit. For the channel to open, each subunit, with closed state S , must change to its "active" state, S' :



where k and k' are the forward and backward rate constants, and G designates an open gate. Then the differential expression for S' will be

$$dS' = k S dt - k' S' dt$$

and if S_0 is the total number of subunits (i.e. $S = S_0 - S'$), then integration, with boundary condition $[S'] = 0$ at $t = 0$, gives

$$[S'] = [S_0] \left\{ \frac{k}{k + k'} \right\} [1 - \exp \{-(k + k')t\}]$$

The probability of a subunit being in the S' state will be $[S']/[S_0]$, so the probability for a gate to be open will be $([S']/[S_0])^n$, i.e. with all the the subunits in the S' form. Calling $[G_0]$ the total number of gates, whether open or closed, we then have for the number of open channels at any one particular time, at a particular membrane potential,

$$[G] = [G_0] \left\{ \left(\frac{k}{k + k'} \right) [1 - \exp \{-(k + k')t\}] \right\}^n \quad (3)$$

Further, they assume that the rate constants are voltage dependent according to

$$k = k_0 \exp \{ez U/k_B T\} \quad ; \quad k' = k'_0 \exp \{ez' U/k_B T\}$$

where k and k' do not depend on the transmembrane potential, U . (U is taken as zero outside, so in the resting state U is -60 or -70 mV). As adjustable parameters, z and z' represent respectively the number of charges per subunit that have to move across the membrane on changing S and S' into an activated transition state during the $S \rightarrow S'$ transformation and its reverse. (The usual meanings apply to e , k_B and T .)

The calcium flow per second, I_{Ca} , that will flow is equal to the number of open gates, $[G]$, times the calcium flux through an individual channel, j . The latter will be governed by the electrodiffusion equation :

$$j = -D(\partial c/\partial x - 2ecE/k_B T)$$

Here D is the calcium diffusion constant, c the molar concentration and E the electric field across the membrane. The continuity equation, $(\partial j/\partial x + \partial c/\partial t = 0)$, then gives us

$$\partial c/\partial t = D \partial^2 c/\partial x^2 - (2e D/k_B T)(\partial(cE)/\partial x)$$

In the steady state, the concentration, c , across the membrane will be constant in time and it is easy to show

that the solution of the differential equation that satisfies c_i for the concentration inside the nerve terminal and c_o for the external concentration, will be

$$j = \frac{\{c_i - c_o \exp(-2eV/k_b T)\}}{\{1 - \exp(-2eV/k_b T)\}} \frac{2e D}{k_b T} \frac{(-V)}{L} \quad (4).$$

L is the thickness of the membrane and D and E are assumed constant along x . The flow decreases with membrane polarization (inside more positive) and changes sign at the calcium equilibrium potential (i.e. when $V = (kT/2e) \ln(c_i/c_o)$, approximately -100 mV, assuming 10^{-7} M Ca inside, 10^{-3} M outside). And so we have

$$I_{Ca} = j A [G]$$

where j and $[G]$ are given as equations (3) and (4) and A is the cross-sectional area of a single channel.

Llinas et al. obtained best fits to their voltage clamp data on the calcium channel with $n = 5$, $k = 2$ /msec, $k' = 1$ /msec, $z = 1$ and $z' = 0$. Figure 6 shows their reconstruction of pre- and postsynaptic events, while Figure 7 is a schematic representation of their proposed calcium channel. On the left, the channel is closed. In the middle, partially open, while on the right a conformational change in all five monomers has been accomplished, allowing calcium to pass.

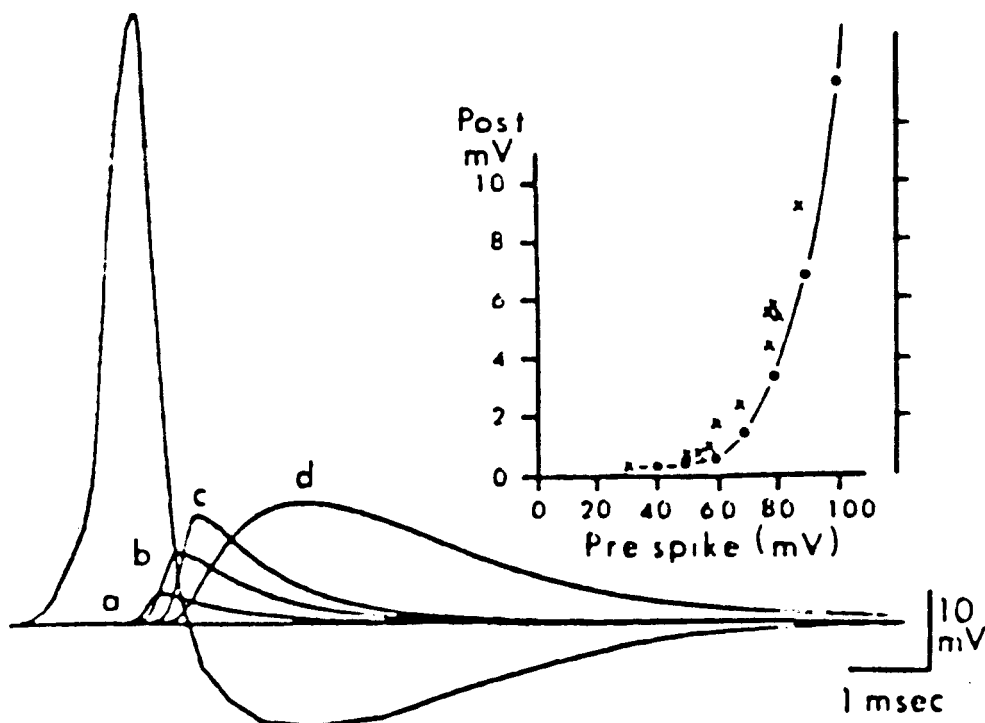


Figure 6. Reconstruction of events at synaptic transmission (From Llinas (1)). Time course of (a) Ca gate formation, (b) calcium current, (c) postsynaptic current and, (d) postsynaptic potential. Inset shows experimental data (x) and model data (•) for various values of presynaptic depolarizations and resultant postsynaptic potentials.

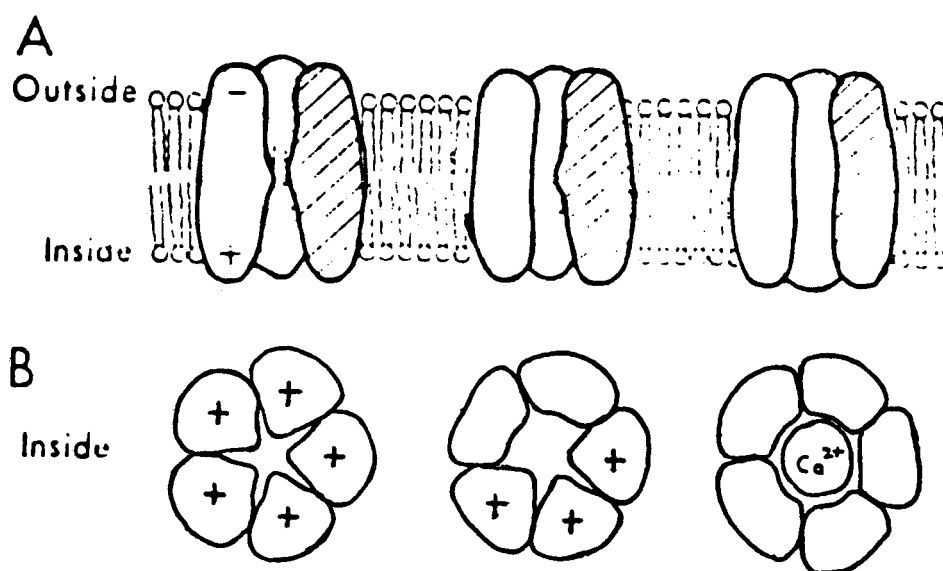


Figure 7. Hypothetical model of calcium channel in various conformations (from Llinas (1)).

In particular, we note that the calcium current flows during the down sweep of the action potential. The magnitude of I_{Ca} is thus related to the amplitude and duration of the action potential, and modulation of these parameters will regulate the amount of neurotransmitter released. Also, because the increase in conductance, $[G]$, comes relatively late during the action potential, it is well matched to the increased driving force for calcium entry.

The relevance of this model to aminopyridine action is clear. By blocking potassium current, the action potential is lengthened and increased calcium entry is produced. The model further predicts an increased synaptic delay--the time between the incoming action potential and the effects on the postsynaptic membrane--in the presence of aminopyridines, which is in fact observed in many experiments.

If the effect of aminopyridines is solely on K^+ channels, what then is to become of the correlation of the adsorption of the aminopyridines to phosphatidylserine with the biological potency? A possible explanation is that the first step (and the rate-limiting one) of the action of the aminopyridines is their capture by the phospholipids. From the membrane surface, then, they are free to diffuse to the potassium channel proteins, with which they could interfere. This "two-stage capture" process--adsorption followed by surface diffusion--is considered by Adam and

Delbruck (38) and also by Berg and Purcell (39). The latter authors stress that efficient collection of molecules is possible simply through the three-dimensional diffusion process, if the receptor sites are evenly dispersed throughout the cell surface. Numerical calculations to verify or refute the two-stage process have not been attempted in this work. In the present case, we are dealing with diffusion through a double layer to complicate the mathematical analysis.

Alternatively, the agreement between binding constants and potency may be fortuitous--the receptor sites on the membrane and in the potassium channels may be similar. Possibly, a certain amount of "hydrophobicity" as measured by our association constants may be important to "anchor" the aminopyridines as they block K^+ channels. (Thomson (12) has proposed this model from his kinetic modeling, where the aminopyridine ring rests in the membrane but the amino substituents block the channel.) Both these pictures suggest that effective aminopyridine analogs that could act as potassium channel blockers in low concentrations would have to have appropriate affinity for lipids.

CHAPTER IV

CAN AMINOPYRIDINES DEPOLARIZE PRESYNAPTIC TERMINALS?

The original hypothesis behind the work, to test the depolarization possibility of aminopyridines, seems now difficult to support. Since the aminopyridines can be effective in low concentrations (e.g. 10 micromolar 3,4-DAP has measurable biological effects (28)), and since they have PS-binding constants of less than 10 per mole, it is difficult to argue that their binding to PS is adequate to sufficiently depolarize the membrane and produce their effects that way. For instance, assuming a Henry's law adsorption isotherm (the limiting case of a Langmuir isotherm at low concentrations), we see less than 10^{-4} of the lipids will be neutralized:

$$\theta = K [AP]$$

i.e. $\theta < 10 (10^{-5}),$

where θ is the fraction of neutralized lipids. Here we have used the bulk aminopyridine concentration. Hille (34) assumes a charge density of 0.14 nm^{-2} for the inner membrane

with a corresponding surface potential of 25 mV (see Figure 6). Even allowing for up to 50 mV, would give corresponding corrections (via the Boltzmann factor) to the bulk concentration of less than 10. This does not help much. At low concentrations, surface potentials will be proportional to surface charge density. A shift of potential of several millivolts, would require, then, θ to be at least on the order of several percent. We conclude that depolarization through neutralization of negatively charged phosphatidylserine lipids cannot account for the facilitatory effects of aminopyridines.

On the other hand, this argument does not rule out the possibility of depolarization effects through binding to the lipid-bound protein. It is conceivable that these binding constants could be as high as 10^3 M^{-1} . In fact, if surface potential could be monitored before and after application of aminopyridine, and changes on the order of several millivolts were observed, then binding constants to proteins could be inferred. Nor is the possibility ruled out that another lipid may be responsible for tightly binding the aminopyridines, although, this would seem unlikely since for other ions, binding constants do not seem to vary drastically from lipid to lipid.

On the other hand, depolarization could account for the excitability effects of aminopyridines. The fact that these effects are not universally reported could reflect

different surface charges on different preparations. Matthews and Wickelgren (40) calculated a depolarization of 5-7 mV for 0.3mM guanidine and measured a decreased threshold potential from 14 to 8 mV. (Guanidine is another facilitatory quaternary compound, which has similar actions to the aminopyridines.) However, the calculation of this depolarization value involved measuring quantal content while varying the external potassium concentration. The shift in potassium concentration, in the presence of guanidine, to reproduce similar quantal contents without guanidine was then used, via the Goldman-Hodgkin-Katz equation (see, for instance, Benedek (24), page 3-222) to calculate the depolarization. But since altering external potassium levels will most likely effect the kinetics of the potassium channels, it is unclear that this value is reliable. Also, these authors assumed the guanidine bound to the outside of the membrane. It seems from our discussion that the binding would have to be to the internal surface to depolarize the cell.

CONCLUSION

The binding constants for a series of aminopyridines to phosphatidylserine vesicles were determined. The order of binding was approximately the same as the order of potency in enhancing synaptic transmission in the presence of reduced calcium concentrations and in normal calcium

concentrations. However, the constants were too low to argue that the aminopyridines could cause their effect by depolarizing the presynaptic membrane by binding to phosphatidylserine at the concentration levels used in the biological experiments. However, they may be able to depolarize it in higher concentrations if there is a substantial difference in surface potential between the outside and inside of the membrane.

A simple model of a calcium channel was discussed that could explain most of the observations of aminopyridine actions. The hypothesis was proposed that the agreement in the order of aminopyridines between their potency in synapses and their association constants with phosphatidylserine membranes may indicate that adsorption to the membrane is the first and rate-limiting step. An alternative possibility would be that the aminopyridines are anchored in the membrane when they are in position to block potassium channels. At the present time it is neither possible to distinguish between these two possibilities nor to verify either of them. Finally, although it cannot be ruled out that the similarities in the sequences are purely fortuitous, we close by hoping that this correlation will find a satisfactory resolution in the future and that the details of aminopyridine action will be made clear.

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APPENDIX A

Gouy-Chapman Theory

It was Helmholtz (1879) who first developed a mathematical theory concerning the electrical double layer --the combination of a charged surface and a layer of oppositely charged ions attracted to it in a solution. Gouy (1909) and Chapman (1913) proposed the idea of a "diffuse double layer", whereby thermal motion tends to disrupt the fixed Helmholtz double layer. Stern (1924) combined the features of both the sharp and the diffuse double layers by postulating a thin layer of bound counterions beyond which lay a diffuse layer. The theory, which applies only to flat surfaces, is essentially unchanged today.

The mathematical framework of the Gouy-Chapman theory is the combination of the Poisson equation to account for the electrostatic attraction to the charged surface, and the Boltzmann equation to take into account the thermal motion of the ions (41, 42). (These two features also form the basis of modern electrolyte theory, and are also used

in plasma and semiconductor physics.)

The potential, U , in the space charge region adjacent to the charged surface will satisfy the Poisson equation in the equilibrium case (MKS units are used throughout),

$$d^2U/dx^2 = -\rho / \epsilon$$

where ρ is the free charge density, $\epsilon = \epsilon_0 \epsilon_r$ is the product of the vacuum permittivity and the dielectric constant respectively, and x represents the distance normal to the charged surface. In thermal equilibrium, with ions of charge q_i and concentration (per cubic meter) c_i , the charge density will be, with appropriate Boltzmann factors (e , k_B and T have their previous meanings):

$$\rho = \sum_i c_i q_i \exp(-q_i U/k_B T)$$

Combining, we then have,

$$\epsilon d^2U/dx^2 = - \sum_i c_i q_i \exp(-q_i U/k_B T)$$

The "Poisson-Boltzmann" equation can be solved with the aid of the substitution, $d^2U/dx^2 = 1/2 d/dU(dU/dx)$, whence,

$$1/2 \epsilon (dU/dx)^2 = - \int dU \sum_i c_i q_i \exp(-q_i U/k_B T)$$

After performing the integral and, with the boundary condition that $dU/dx = 0$ when $U = 0$ (at $x = \infty$), we get

$$\epsilon (dU/dx)^2 = 2k_B T \sum_i c_i \{ \exp(-q_i U/k_B T) - 1 \}$$

Since at the surface, $\sigma = -\epsilon dU/dx$, we obtain from the above equation, the Grahame equation, which relates the surface charge density, σ , to the surface potential, U_0 ,

$$\sigma = (U_0/|U_0|) (2k_B T \epsilon)^{1/2} \left[\sum_i c_i \{ \exp(-q_i U_0/k_B T) - 1 \} \right]^{1/2}$$

To proceed further, we restrict ourselves to a z:z electrolyte, concentration c. There will be only two terms in the sum and the Grahame equation reduces to

$$\sigma = (U_0/|U_0|) (8k_B T c \epsilon)^{1/2} \sinh(zeU_0/2k_B T) \quad (A1)$$

A second integration of the Poisson-Boltzmann equation can be performed, using the relation $\int dp/\sinh p = \ln [\tanh(p/2)]$, leaving

$$\ln \left[\frac{\tanh(zeU/4k_B T)}{\tanh(zeU_0/4k_B T)} \right] = x / \left[(\epsilon k_B T) / (2e^2 z^2 c) \right]^{1/2}$$

or, defining the Debye length, $\lambda_D = [(\epsilon k_B T) / (2e^2 z^2 c)]^{1/2}$,

$$\tanh(zeU/4k_B T) = \tanh(zeU_0/4k_B T) \exp(-x/\lambda_D) \quad (A2).$$

For $zeU/k_B T \ll 1$, (i.e. $U \ll 25$ mV, for a monovalent salt at room temperature), (A2) reduces to the purely exponential decay,

$$U = U_0 \exp(-x/\lambda_D)$$

with a decay length, λ_D , (Debye screening length) of, for

instance, 10 angstroms for a decimolar univalent electrolyte at 25 °C. The Debye length gives the formal expression for our previously ill-defined "thickness" of the double layer in chapter two. In the case of small potentials, a simple interpretation is possible. The Debye length is the distance for the potential to drop to $1/e$ of its surface value.

Abraham-Schrauner has also obtained an analytical solution to the planar Poisson-Boltzmann equation for the case of a 2:1 electrolyte (43). Bentz and Nir (44) have generalized her equation to accommodate any combination of mono and divalent ions. Their equation (A11) was used in determining the binding constant for calcium. (In our case, because we also had in some of our solutions small amounts of trivalent buffer, a numerical program was also written to solve the Poisson-Boltzmann equation for any combination of ions of any charge. However, the program was slow and proved not to be necessary.)

Before leaving the electrical double layer, we note some of the approximations inherent in the theory. First we mention that many important physicists and physical chemists (Fowler, Guggenheim, Onsager, Kramers, Kirkwood and Casimir) have worked on the problem of an inconsistency in the Poisson-Boltzmann equation, which arises when the average potential in the Poisson equation is equated with the "potential of mean force" in the Boltzmann relation.

(See Overbeek and Lijklema (22) for original references , also (45).) This is so because $q_i U(x)$ ought to be the work done to bring ion i to position x , but when the ion is at x , it will influence the distribution of ions in its neighborhood, so that the potential at x will deviate from the mean potential $U(x)$. Verwey and Overbeek (46) state that "for large U , the determination of the mean value of c from U is strictly speaking not allowed." However, the general consensus is that in the full non-linear Poisson-Boltzmann equation the error introduced will be small when the flat surface bears many charges and the ionic concentrations do not rise to values at which deviations from the Debye-Huckel laws become considerable.

Also implicit in the Gouy-Chapman theory is the neglect of discrete charge effects--we deal only with "smeared" charge densities (47). Furthermore, the ions are treated as point charges. Thus we ignore that they have a finite volume and an individual polarizability (and perhaps a permanent dipole moment), and that they will displace an equal volume of solvent molecules which will also have a polarizability and dipole moment (48).

Polarization effects at the interface (e.g. from restrictions on solvent orientation (49) or from the lipid head groups (27)) are also ignored. Finally, the dielectric constant is assumed not to vary as the interface is approached (27, 49). However, despite its shortcomings

and the likelihood that some of the approximations compensate for each other, we have found that the basic Gouy-Chapman-Stern theory gives an adequate description to our data.

APPENDIX B

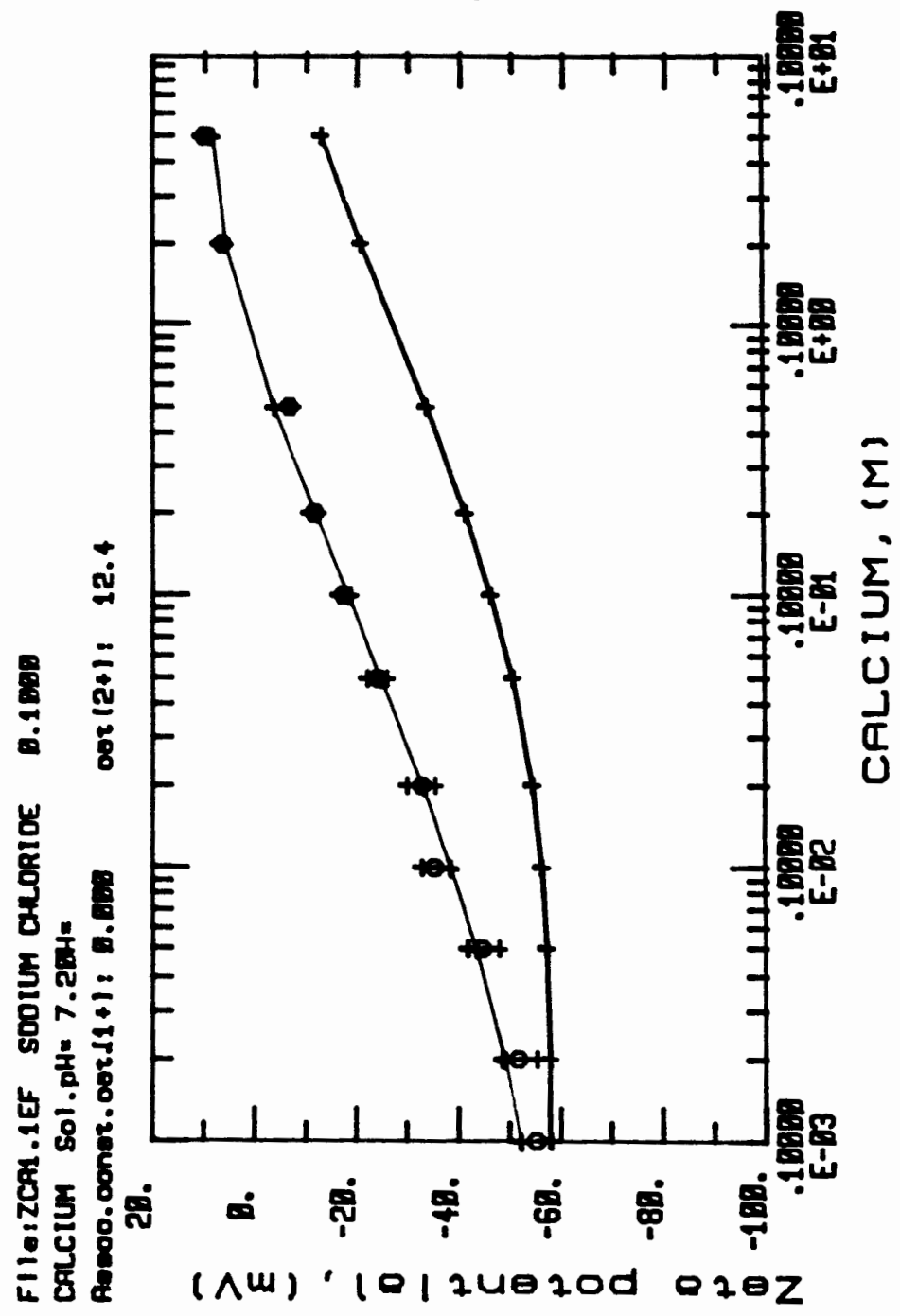


Figure B. Adsorption isotherm for calcium.

Program DIVKAI 05-MAY-86 16:19:04
 Experimental results: data file ZCA1.POS
 Lipid:PHOSPHA SERINE
 OLD ALEX'S DATA/biophys.soc.poster
 Membrane active molecule:CALCIUM
 Fractional charge= 2.00
 Background electrolyte:SODIUM CHLORIDE 0.100 mole/liter
 Buffer:MOPS Concentration= 0.001 mole/liter
 Solution pH: 7.20

Analytical results:

Lipid density= 0.1538E+19(1/m**2)
 Lipid saturation parameter, phi= 1.000
 Assoc.const.,background cation= 1.00 (liter/mole)
 Assoc.const.,singly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Assoc.const.,doubly charged molecules= 12.1 +/- 1.27 (liter/mole)
 Chi-square = 2.726

X-molecule conc.(M)	Zeta pot.(expt)+/-st.dev.(mV)	Zeta pot.(theor) (mV)
0.000000	-60.10 +/- 4.00	-58.66
0.000100	-55.20 +/- 3.10	-52.66
0.000200	-51.90 +/- 3.40	-49.41
0.000500	-44.80 +/- 3.00	-43.83
0.001000	-35.40 +/- 2.70	-38.79
0.002000	-32.70 +/- 2.60	-33.20
0.005000	-24.10 +/- 1.90	-25.15
0.010000	-17.60 +/- 1.30	-18.75
0.020000	-11.60 +/- 0.90	-12.26
0.050000	-6.90 +/- 0.70	-4.03
0.100000	0.00 +/- 1.00	1.40
0.200000	6.60 +/- 0.50	5.52
0.500000	9.90 +/- 0.85	8.06

TABLE II

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
FOR CALCIUM

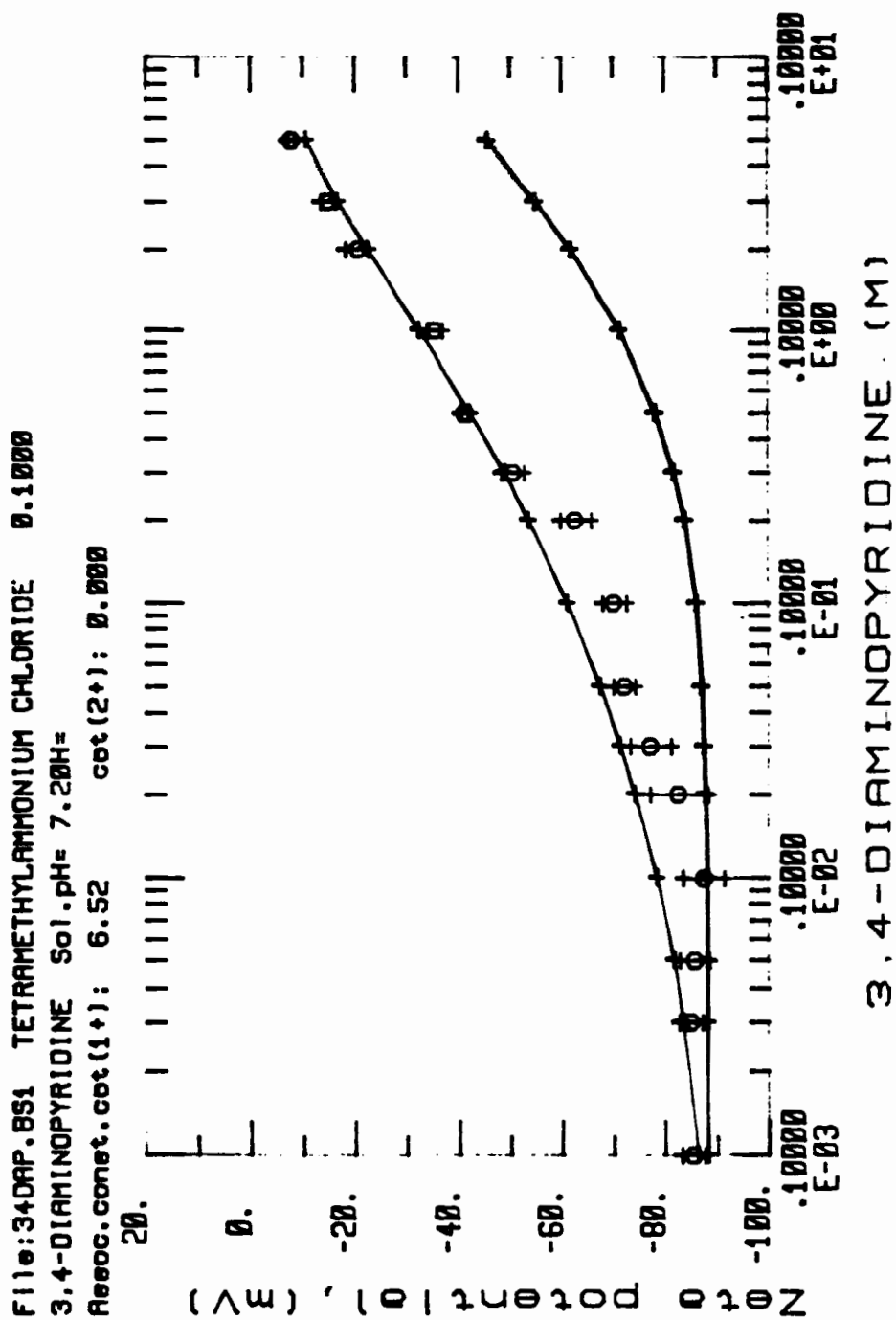


Figure 9. Adsorption isotherm for 3,4-diaminopyridine.

Program DIUKA1 05-MAY-86 16:42:31
 Experimental results: data file 34DAP.BS1
 Lipid:PHOSPHAT SERINE
 COMBINED PSM+ALEX DATA, BIOPHYSICAL SOC (25 DEGREES)
 Membrane active molecule:3,4-DIAMINOPYRIDINE
 Fractional charge= 1.00
 Background electrolyte:TETRAMETHYLAMMONIUM CHLORIDE 0.100 mole/liter
 Buffer:HOPS Concentration= 0.001 mole/liter
 Solution pH: 7.20

Analytical results:
 Lipid density= $0.1538E+19(1/m^{**2})$
 Lipid saturation parameter, phi= 1.000
 Assoc.const.,background cation= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 6.52 +/- 0.980 (liter/mole)
 Assoc.const.,doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 4.332

X-molecule conc.(M)	Zeta pot.(exper)+/-st.dev.(mV)	Zeta Pot.(theor) (mV)
0.000000	-87.50 +/- 1.80	-88.40
0.000100	-85.40 +/- 2.00	-86.41
0.000300	-85.10 +/- 2.10	-83.76
0.000500	-85.70 +/- 2.80	-81.88
0.001000	-87.50 +/- 3.90	-78.60
0.002000	-82.70 +/- 5.40	-74.44
0.003000	-77.30 +/- 3.90	-71.58
0.005000	-72.30 +/- 2.20	-67.52
0.010000	-70.10 +/- 2.30	-61.17
0.020000	-62.70 +/- 3.10	-53.76
0.030000	-50.40 +/- 2.20	-48.90
0.050000	-41.20 +/- 0.90	-42.24
0.100000	-35.30 +/- 1.50	-32.41
0.200000	-20.60 +/- 2.30	-22.34
0.300000	-15.00 +/- 1.40	-16.82
0.500000	-7.90 +/- 0.70	-10.78

TABLE III

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 3,4-DIAMINOPYRIDINE

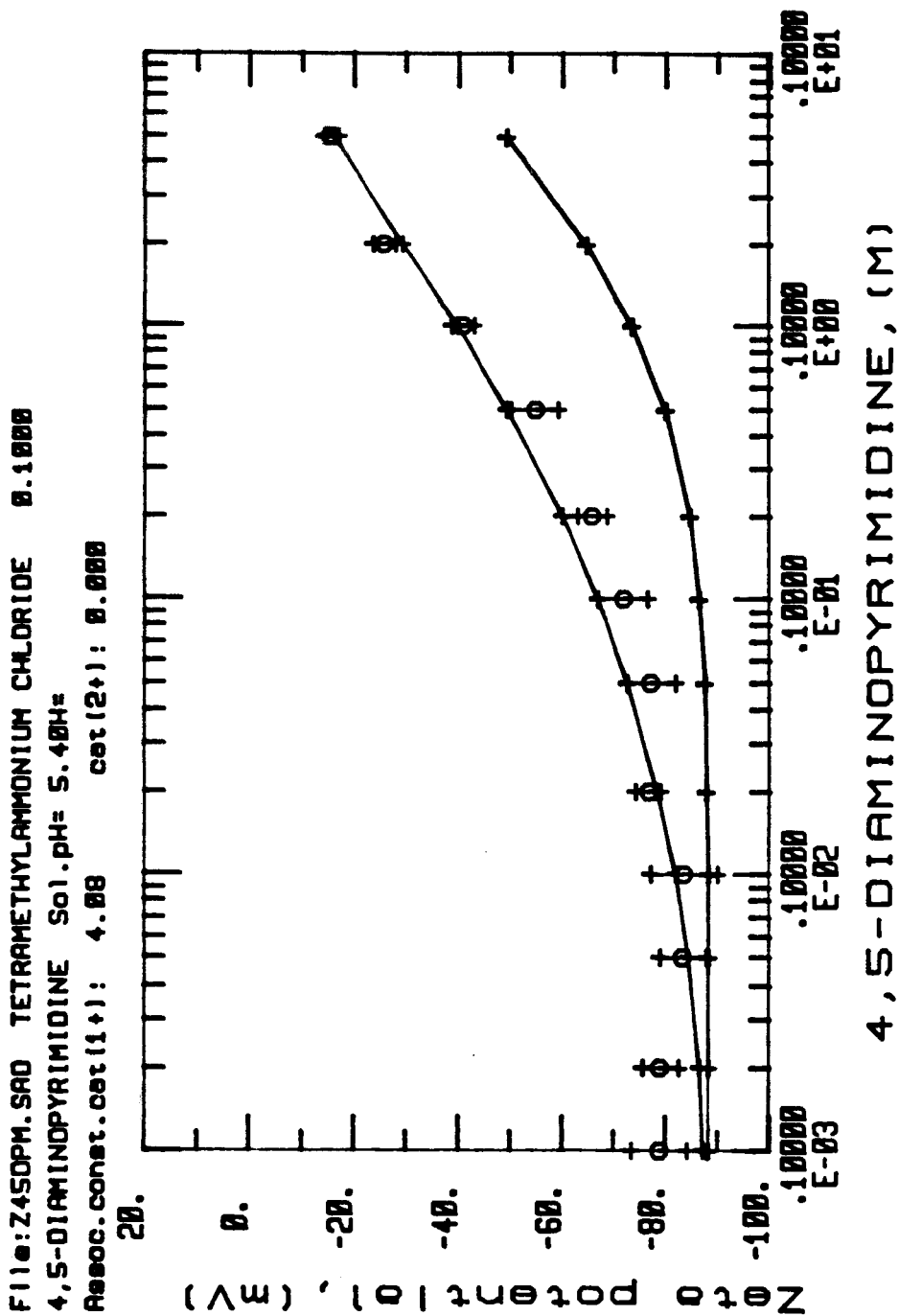


Figure 10. Adsorption isotherm for 4,5-diaminopyrimidine (pH 5.4).

Program DIVKA1 05-MAY-86 18:04:13
 Experimental results: data file Z45DPM.SAD
 Lipid:PHOSPHAT SERINE
 PERFORMED BY CW 26-FEB-86
 Membrane active molecule:4,5-DIAMINOPYRIMIDINE
 Fractional charge= 0.810
 Background electrolyte:TETRAMETHYLAMMONIUM CHLORIDE 0.100 mole/liter
 Buffer:BORATE/CITRATE/PHOSPHATE--B-3 Concentration= 0.004 mole/liter
 Solution pH: 5.40

Analytical results:

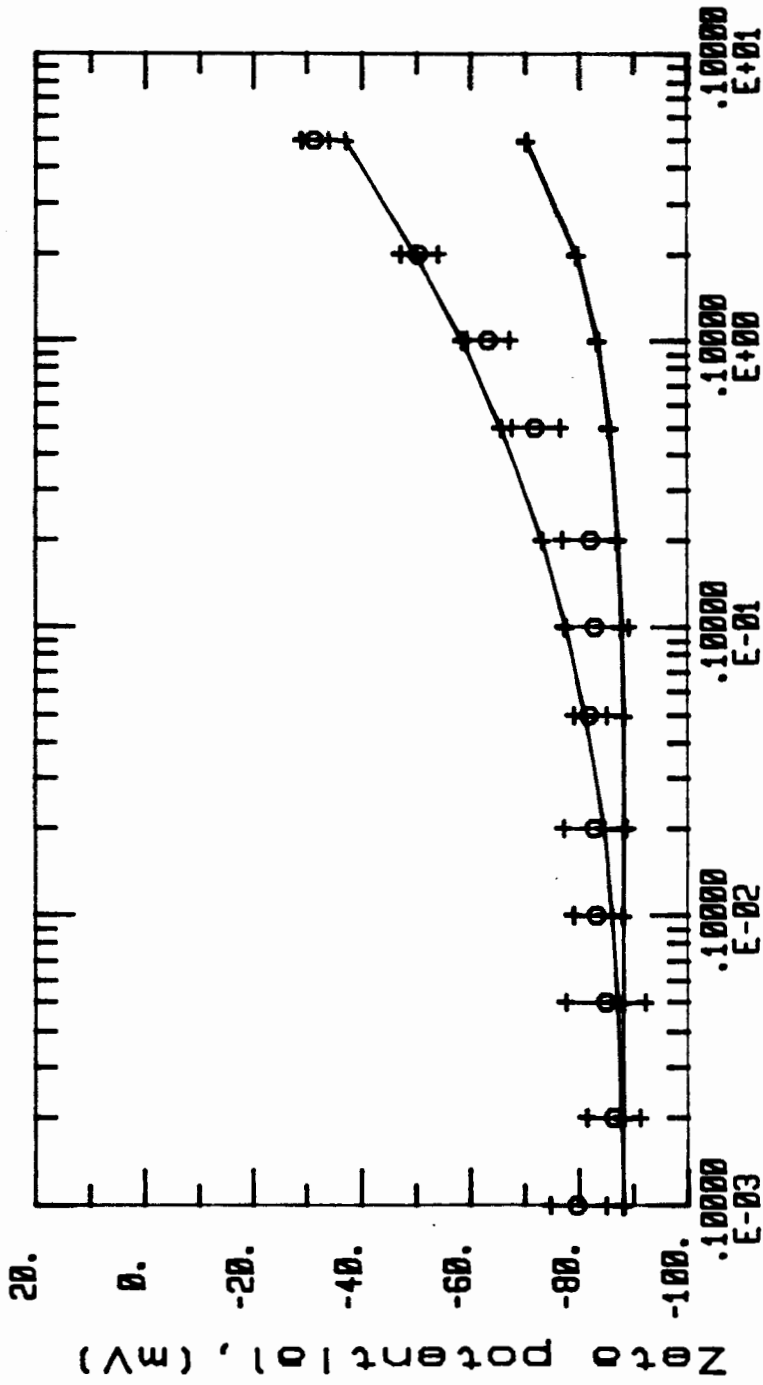
Lipid density= 0.1538E+19(1/m**2)
 Lipid saturation parameter, phi= 1.000
 Assoc.const.,background cation= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 4.08 +/- 1.06 (liter/mole)
 Assoc.const.doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 1.507

X-molecule conc.(M)	Zeta Pot.(expr)+/-st.dev.(mV)	Zeta Pot.(theor) (mV)
0.000000	-81.40 +/- 5.30	-88.40
0.000100	-79.00 +/- 5.30	-87.28
0.000200	-79.00 +/- 3.40	-86.35
0.000500	-83.40 +/- 4.60	-84.22
0.001000	-83.60 +/- 6.50	-81.72
0.002000	-76.70 +/- 2.20	-78.35
0.005000	-77.10 +/- 4.70	-72.47
0.010000	-71.70 +/- 4.90	-66.85
0.020000	-65.70 +/- 2.90	-60.11
0.050000	-54.50 +/- 4.60	-49.28
0.100000	-40.60 +/- 2.20	-39.65
0.200000	-25.60 +/- 2.10	-29.22
0.500000	-15.50 +/- 1.20	-16.02

TABLE IV

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 4-5-DIAMINOPYRIDINE (PH 5.4)

File:Z45DPM.TAD TETRAMETHYLAMMONIUM CHLORIDE 0.1000
 4,5-DIAMINOPYRIMIDINE Sol.pH= 6.60H=
 Assoc.const.cat(1+): 3.48 cat(2+): 0.000



4,5-DIAMINOPYRIMIDINE, (M)

Figure 11. Adsorption isotherm for 4,5-diaminopyrimidine (pH 6.6).

Program DIVKA1 05-MAY-86 18:25:19
 Experimental results: data file Z45DPM.TAD
 Lipid:PHOSPHAT SERINE
 PERFORMED BY C.WRIGHT 1 & 2 MARCH-86
 Membrane active molecule:4,5-DIAMINOPYRIMIDINE
 Fractional charge= 0.210
 Background electrolyte:TETRAMETHYLAMMONIUM CHLORIDE 0.100 mole/liter
 Buffer:BORATE/CITRATE/PHOSPHATE--B-3 Concentration= 0.004 mole/liter
 Solution pH: 6.60

Analytical results:

Lipid density= 0.1538E+19(1/m**2)
 Lipid saturation parameter, phi= 1.000
 Assoc.const.,background cation= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 3.48 +/- 1.47 (liter/mole)
 Assoc.const.,doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 1.316

X-molecule conc.(M)	Zeta Pot.(expr)+/-st.dev.(mV)	Zeta Pot.(theor) (mV)
0.000000	-88.50 +/- 4.90	-88.40
0.000100	-79.80 +/- 5.20	-88.13
0.000200	-86.40 +/- 4.90	-87.87
0.000500	-84.90 +/- 7.30	-87.16
0.001000	-83.40 +/- 4.60	-86.15
0.002000	-83.00 +/- 5.70	-84.53
0.005000	-82.00 +/- 3.10	-81.21
0.010000	-83.00 +/- 5.90	-77.68
0.020000	-82.10 +/- 5.10	-73.18
0.050000	-72.00 +/- 4.40	-65.63
0.100000	-63.30 +/- 4.00	-58.54
0.200000	-50.40 +/- 3.40	-50.13
0.500000	-31.40 +/- 2.50	-37.08

TABLE U

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 4,5-DIAMINOPYRIMIDINE (PH 6.6)

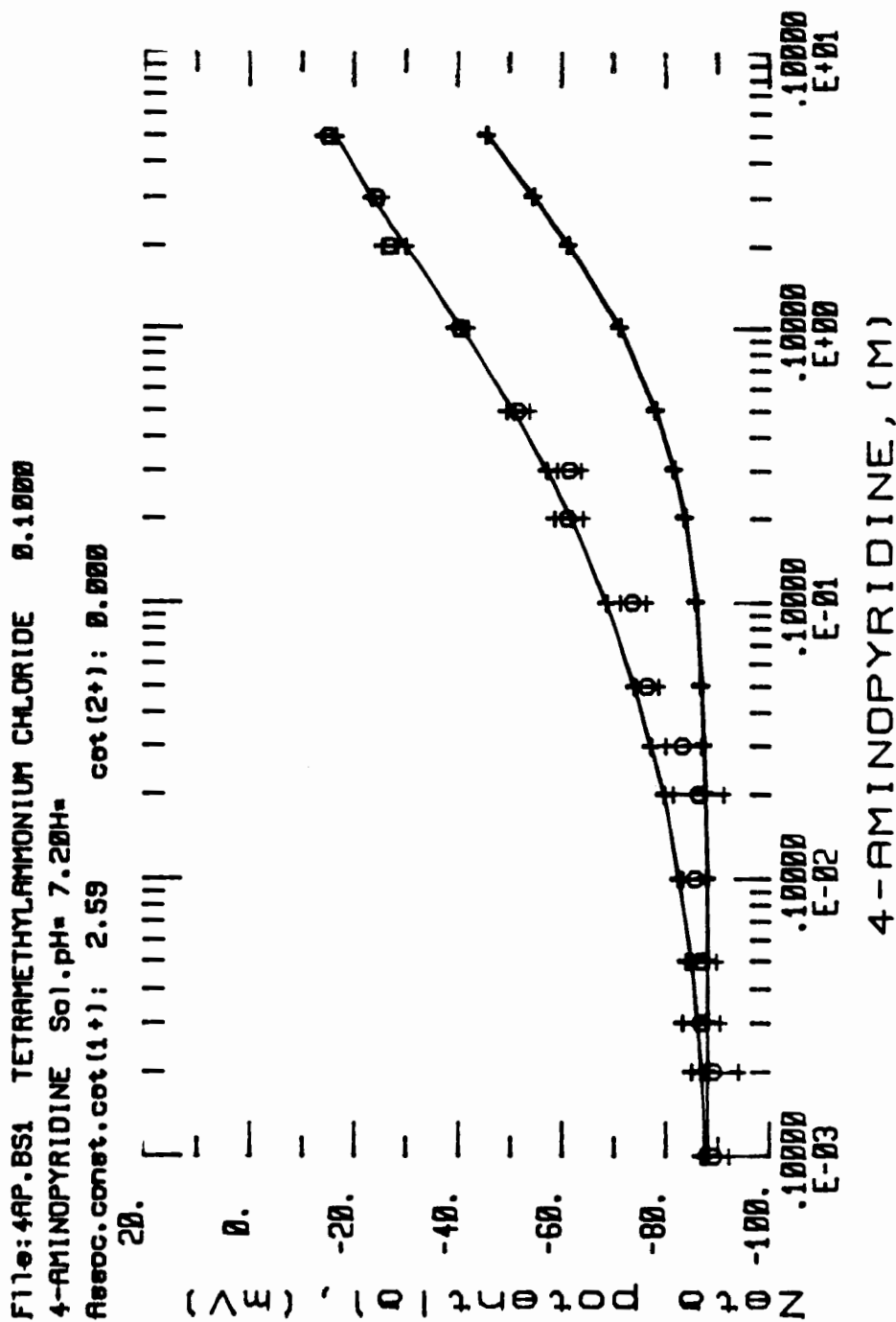


Figure 12. Adsorption isotherm for 4-aminopyridine.

Program DIVKA1 05-MAY-86 17:01:04
 Experimental results: data file 4AP.BS1
 Lipid:PHOSPHAT SERINE
 COMBINED PSM+ALEX DATA, 5MG/100 ML PS, 25 DEGREES, BIOPHYSICAL SOC
 Membrane active molecule:4-AMINOPYRIDINE
 Fractional charge= 1.00
 Background electrolyte:TETRAMETHYLAMMONIUM CHLORIDE 0.100 mole/liter
 Buffer:MOPS Concentration= 0.001 mole/liter
 Solution pH: 7.20

Analytical results:
 Lipid density= 0.1538E+19(1/m**2)
 Lipid saturation Parameter, phi= 1.000
 Assoc.const.,background cation= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 2.59 +/- 0.465 (liter/mole)
 Assoc.const.doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 1.583

X-molecule conc.(M)	Zeta pot.(expr)+/-st.dev.(mV)	Zeta Pot.(theor) (mV)
0.000000	-88.40 +/- 2.60	-88.40
0.000100	-89.50 +/- 2.70	-87.50
0.000200	-89.40 +/- 4.50	-86.73
0.000300	-86.80 +/- 3.60	-86.06
0.000500	-86.90 +/- 2.90	-84.89
0.001000	-85.60 +/- 2.30	-82.66
0.002000	-86.40 +/- 4.90	-79.55
0.003000	-83.40 +/- 3.30	-77.30
0.005000	-76.40 +/- 2.20	-73.96
0.010000	-73.60 +/- 2.60	-68.47
0.020000	-61.20 +/- 2.70	-61.75
0.030000	-61.30 +/- 2.30	-57.17
0.050000	-51.40 +/- 2.20	-50.70
0.100000	-40.50 +/- 1.30	-40.75
0.200000	-26.70 +/- 1.40	-29.95
0.300000	-24.30 +/- 1.10	-23.68
0.500000	-14.90 +/- 0.90	-16.37

TABLE UI

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 4-AMINOPYRIDINE

File: Z3AP.PAD TMA CHLORIDE 0.1000
 3-AMINOPYRIDINE Sol. pH= 5.40H=
 Assoc. const. cot (1+): 1.40 cot (2+): 0.000

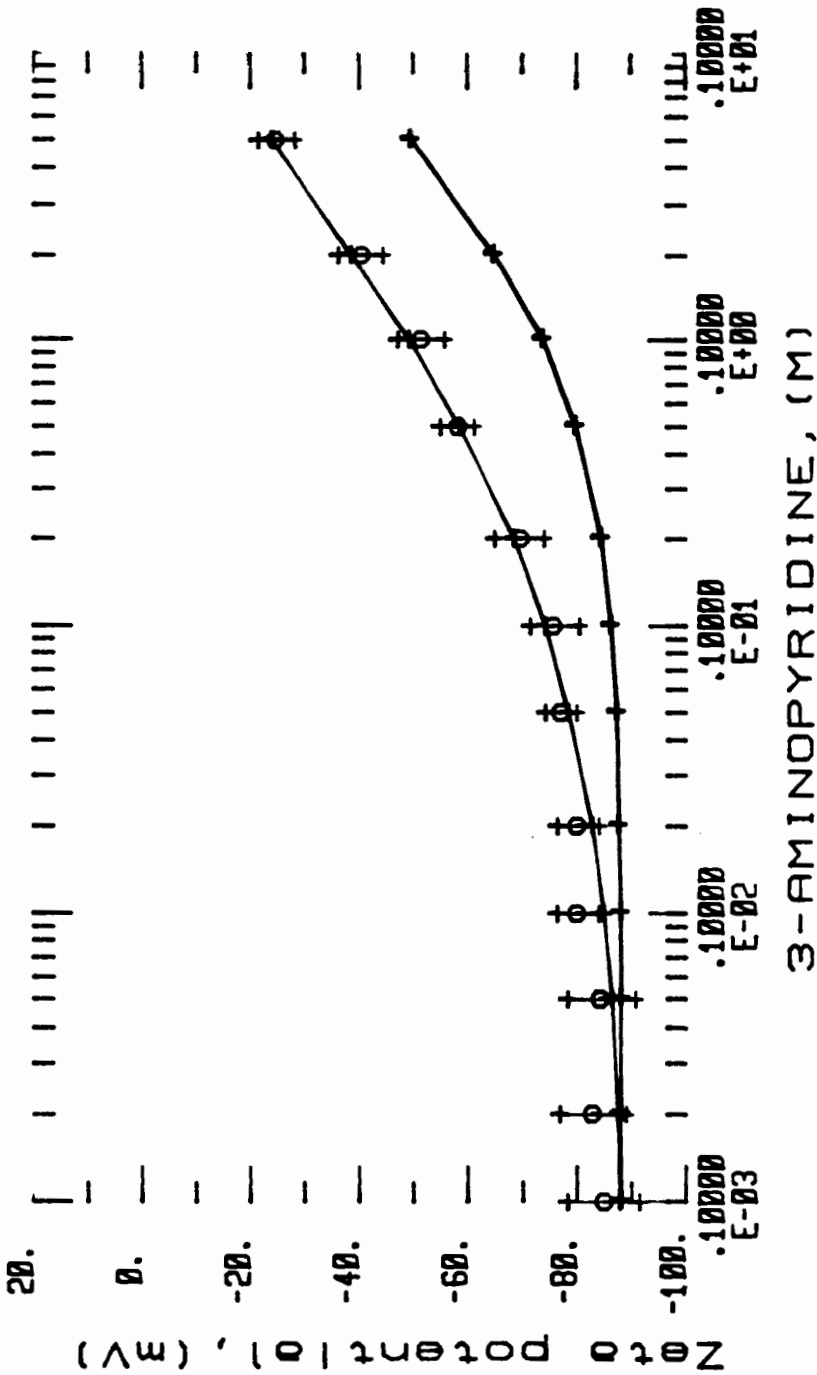


Figure 13. Adsorption isotherm for 3-aminopyridine (pH 5.4)

Program DIUKAL 05-MAY-86 17:08:38
 Experimental results: data file Z3AP.PAD
 Lipid:PHOSPHAT SERINE
 PERFORMED 2/1/86 BY CWRIGHT
 Membrane active molecule:3-AMINOPYRIDINE
 Fractional charge= 0.790
 Background electrolyte:TMA CHLORIDE 0.100 mole/liter
 Buffer:BORATE/CITRATE/PHOSPHATE Concentration= 0.002 mole/liter
 Solution pH: 5.40

Analytical results:
 Lipid density= 0.1538E+19(1/m**2)
 Lipid saturation parameter, phi= 1.000
 Assoc.const.,background cation= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 1.40 +/- 0.669 (liter/mole)
 Assoc.const.doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 0.3553

X-molecule conc.(M)	Zeta Pot.(expr)+/-st.dev.(mV)	Zeta Pot.(theor) (mV)
0.000000	-85.00 +/- 6.20	-88.40
0.000100	-84.90 +/- 6.60	-87.99
0.000200	-83.00 +/- 6.00	-87.60
0.000500	-84.50 +/- 6.10	-86.57
0.001000	-80.10 +/- 3.80	-85.18
0.002000	-80.20 +/- 3.80	-83.03
0.005000	-77.20 +/- 2.90	-78.79
0.010000	-75.90 +/- 4.60	-74.32
0.020000	-69.50 +/- 4.30	-68.58
0.050000	-58.10 +/- 3.00	-58.70
0.100000	-51.40 +/- 4.20	-49.32
0.200000	-40.20 +/- 4.00	-38.57
0.500000	-24.80 +/- 3.50	-23.76

TABLE VII

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 3-AMINOPYRIDINE (PH 5.4)

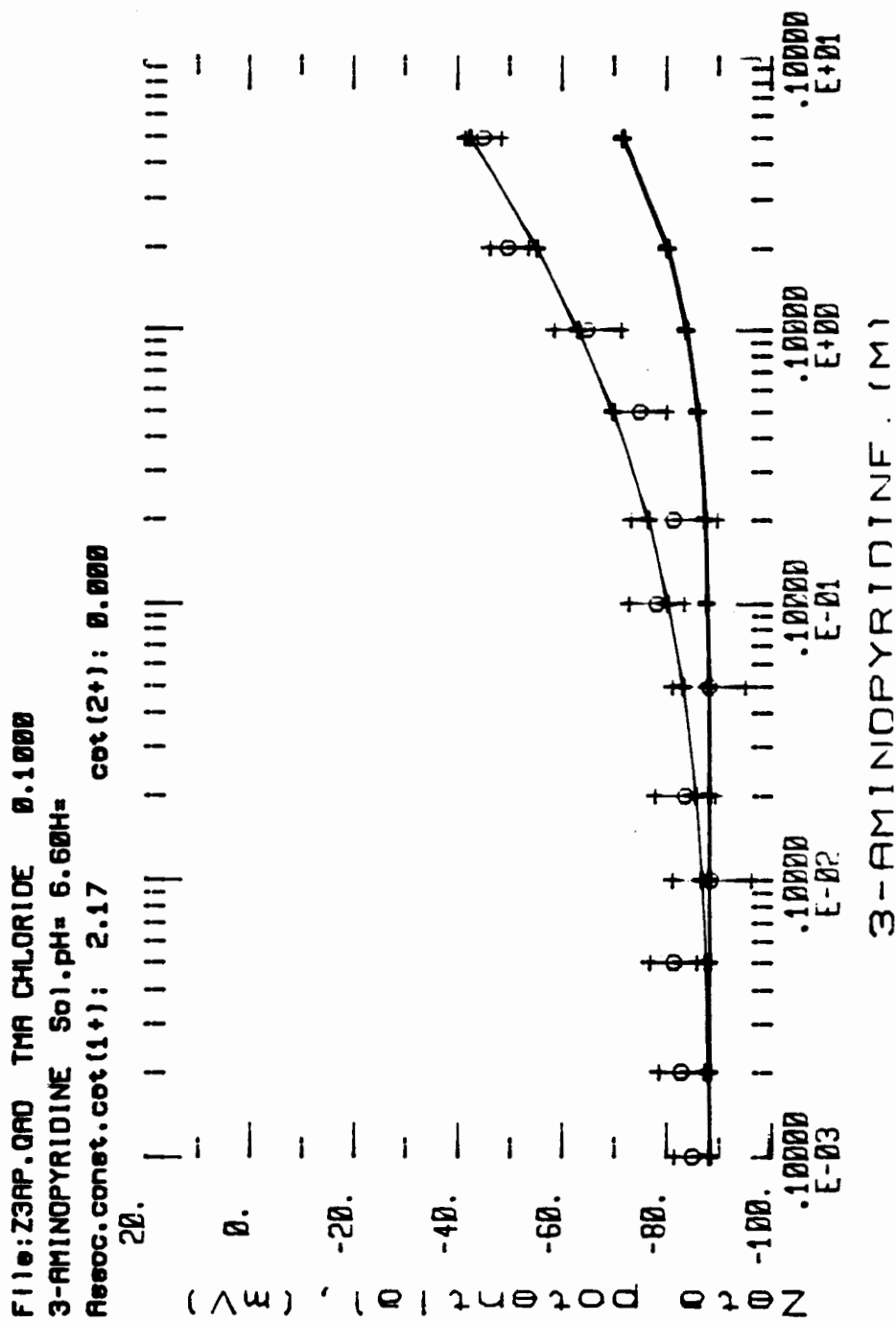


Figure 14. Adsorption isotherm for 3-aminopyridine (pH 6.6).

Program DIUKA1 05-MAY-86 17:53:53
 Experimental results: data file Z3AP.GAD
 Lipid:PHOSPHAT SERINE
 PERFORMED 3+4 FEB.86 BY CW
 Membrane active molecule:3-AMINOPYRIDINE
 Fractional charge= 0.200
 Background electrolyte:TMA CHLORIDE 0.100 mole/liter
 Buffer:BORATE/CITRATE/PHOSPHATE--B-3 Concentration= 0.004 mole/liter
 Solution pH: 6.60

Analytical results:

Lipid density= 0.1538E+19(1/m**2)
 Lipid saturation parameter, phi= 1.000
 Assoc.const.,background cation= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 2.17 +/- 1.30 (liter/mole)
 Assoc.const.doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 0.7920

X-molecule conc.(M)	Zeta Pot.(expr)+/-st.dev.(mV)	Zeta Pot.(thcor) (mV)
0.000000	-84.70 +/- 5.90	-88.40
0.000100	-84.90 +/- 3.40	-88.24
0.000200	-82.90 +/- 4.30	-88.09
0.000500	-81.40 +/- 4.40	-87.65
0.001000	-88.50 +/- 7.50	-86.99
0.002000	-83.60 +/- 5.70	-85.87
0.005000	-88.10 +/- 6.90	-83.35
0.010000	-78.10 +/- 5.10	-80.48
0.020000	-81.40 +/- 8.20	-76.65
0.050000	-75.10 +/- 5.10	-69.93
0.100000	-65.00 +/- 6.40	-63.39
0.200000	-49.70 +/- 3.80	-55.43
0.500000	-44.90 +/- 3.30	-42.64

TABLE VIII

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 3-AMINOPYRIDINE (PH 6.6)

File: Z2AP.VAO TETRAMETHYLAMMONIUM CHLORIDE 0.1000
 2-AMINOPYRIDINE Sol. pH= 6.20M=
 Assoc. const. cat (1+): 1.72 cat (2+): 0.000

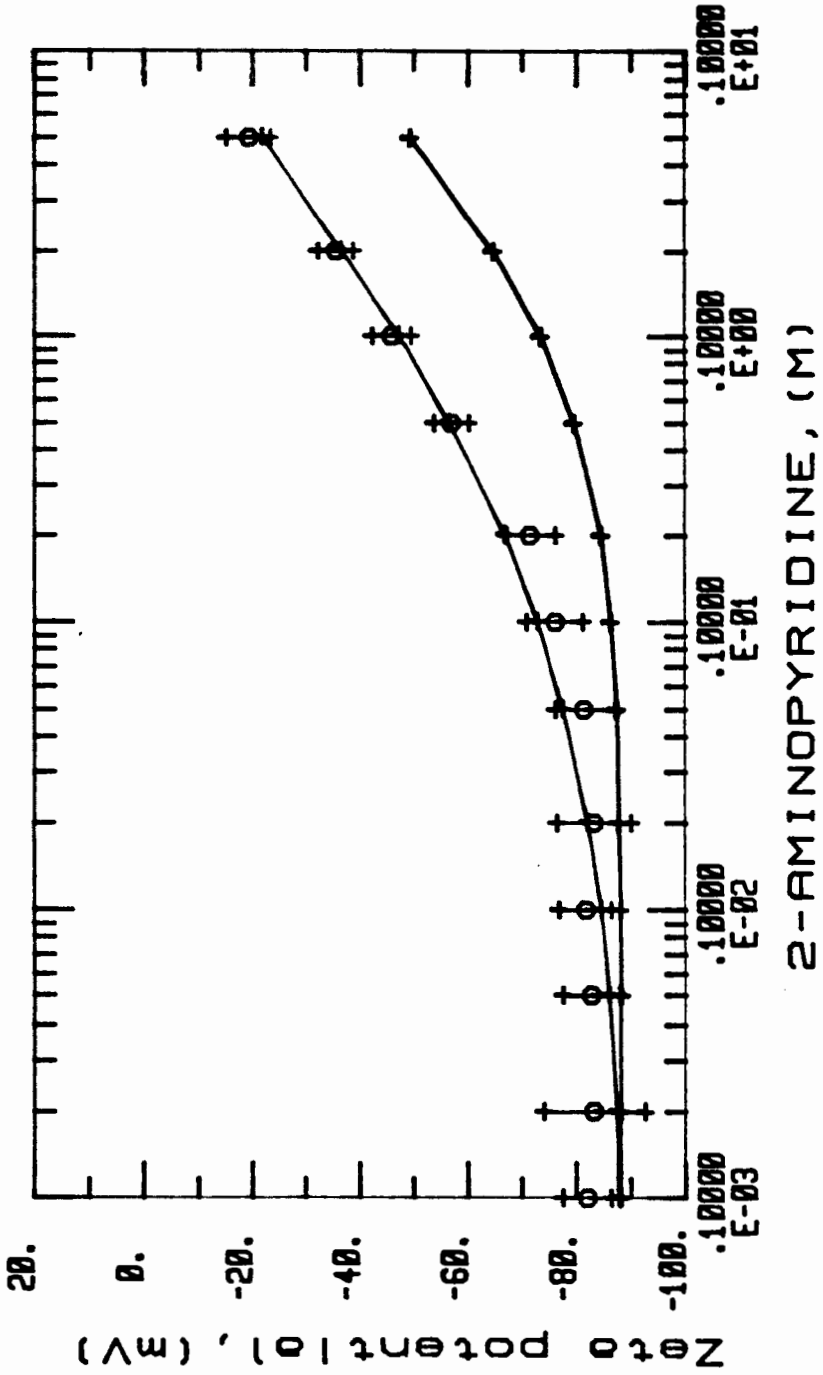


Figure 15. Adsorption isotherm for 2-aminopyridine (pH 6.2).

Program DIVKA1 05-MAY-86 18:34:05
 Experimental results: data file ZZAP.WAD
 Lipid:PHOSPHAT SERINE
 PERFORMED BY CW 24-MAR-86
 Membrane active molecule:2-AMINOPYRIDINE
 Fractional charge= 0.820
 Background electrolyte:TETRAMETHYLAMMONIUM CHLORIDE 0.100 mole/liter
 Buffer:BORATE/CITRATE/PHOSPHATE B-3 Concentration= 0.004 mole/liter
 Solution pH: 6.20

Analytical results:

Lipid density= 0.1538E+19(1/M**2)
 Lipid saturation parameter, Phi= 1.000
 Assoc.const.,background cation= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 1.72 +/- 0.790 (liter/mole)
 Assoc.const.,doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 0.4578

X-molecule conc.(M)	Zeta Pot.(expr)+/-st.dev.(mV)	Zeta Pot.(thcor) (mV)
0.000000	-82.10 +/- 3.80	-88.40
0.000100	-82.10 +/- 4.40	-87.88
0.000200	-83.20 +/- 9.20	-87.41
0.000500	-82.90 +/- 5.50	-86.18
0.001000	-81.70 +/- 4.70	-84.57
0.002000	-83.20 +/- 6.90	-82.16
0.005000	-81.60 +/- 5.60	-77.54
0.010000	-76.00 +/- 5.10	-72.79
0.020000	-71.60 +/- 4.40	-66.79
0.050000	-56.90 +/- 3.20	-56.61
0.100000	-45.70 +/- 3.60	-47.09
0.200000	-35.40 +/- 3.10	-36.31
0.500000	-19.10 +/- 4.00	-21.78

TABLE IX

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 2-AMINOPYRIDINE (PH 6.2)

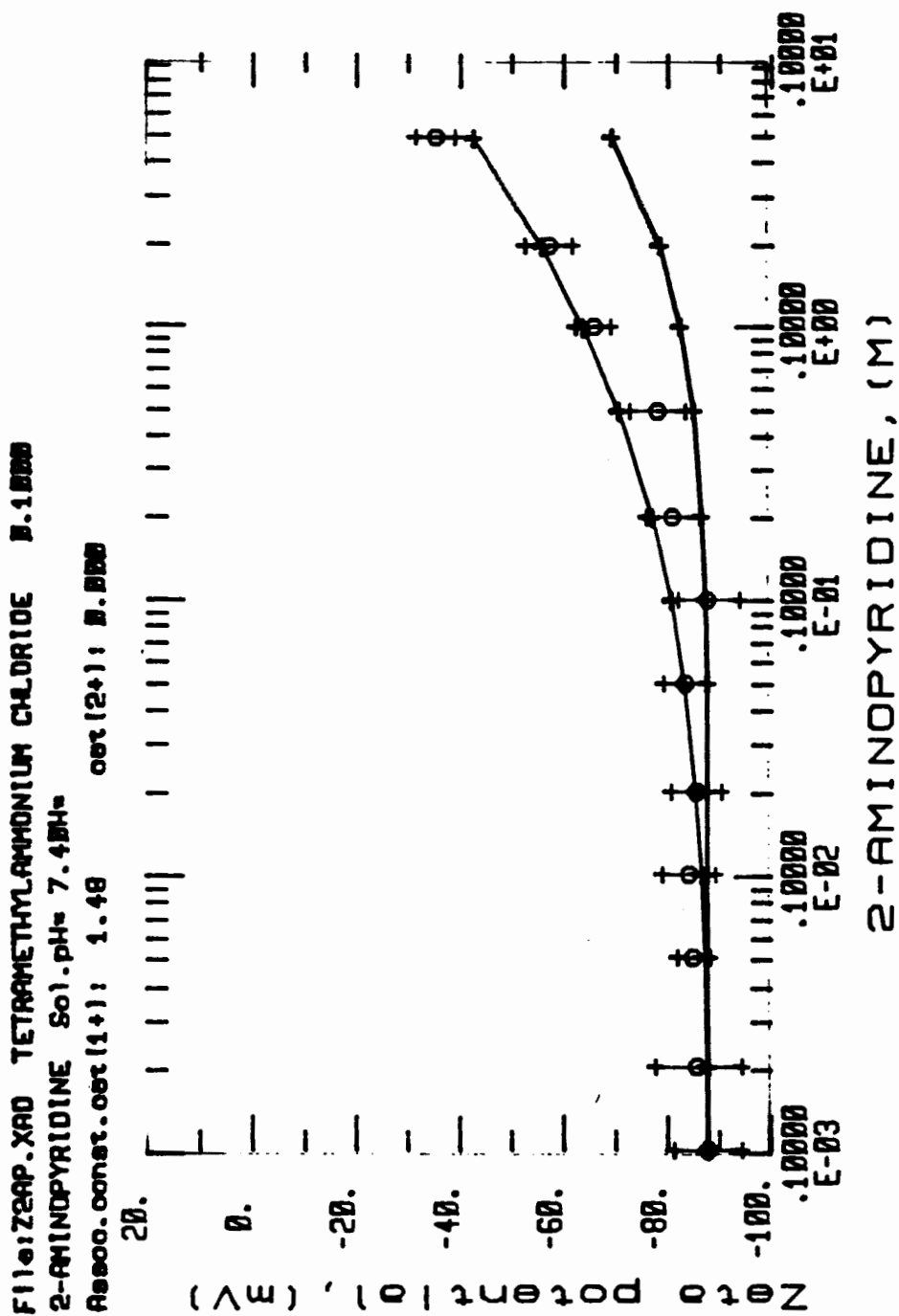


Figure 16. Adsorption isotherm for 2-aminopyridine (pH 7.4).

Program DIUKA1 05-MAY-86 18:41:46
 Experimental results: data file Z2AP.XAD
 Lipid:PHOSPHAT SERINE
 PERFORMED BY CW 25-MAR-86
 Membrane active molecule:2-AMINOPYRIDINE
 Fractional charge= 0.220
 Background electrolyte:TETRAMETHYLAMMONIUM CHLORIDE 0.100 mole/liter
 Buffer:BORATE/CITRATE/PHOSPHATE B-3 Concentration= 0.004 mole/liter
 Solution pH: 7.40

Analytical results:

Lipid density= $0.1538E+19(1/m^{**2})$
 Lipid saturation parameter, phi= 1.000
 Assoc.const.,background cations= 0.000 (liter/mole)
 Assoc.const.,singly charged molecules= 1.47 +/- 0.886 (liter/mole)
 Assoc.const.,doubly charged molecules= 0.000 +/- 0.000 (liter/mole)
 Chi-square = 0.7354

X-molecule conc.(M)	Zeta Pot.(expt)+/-st.dev.(mV)	Zeta Pot.(theor) (mV)
0.000000	-99.30 +/- 6.00	-88.40
0.000100	-88.10 +/- 6.60	-88.28
0.000200	-86.30 +/- 8.40	-86.15
0.000500	-85.40 +/- 3.30	-87.80
0.001000	-84.20 +/- 5.20	-87.25
0.002000	-86.00 +/- 4.80	-86.29
0.005000	-84.30 +/- 4.30	-84.07
0.010000	-88.30 +/- 5.90	-81.45
0.020000	-81.80 +/- 5.20	-77.84
0.050000	-78.60 +/- 5.30	-71.32
0.100000	-66.30 +/- 3.50	-64.83
0.200000	-57.80 +/- 4.40	-56.78
0.500000	-35.90 +/- 3.90	-42.69

TABLE X

THEORETICAL VS EXPERIMENTAL ZETA POTENTIALS
 FOR 2-AMINOPYRIDINE (PH 7.4)

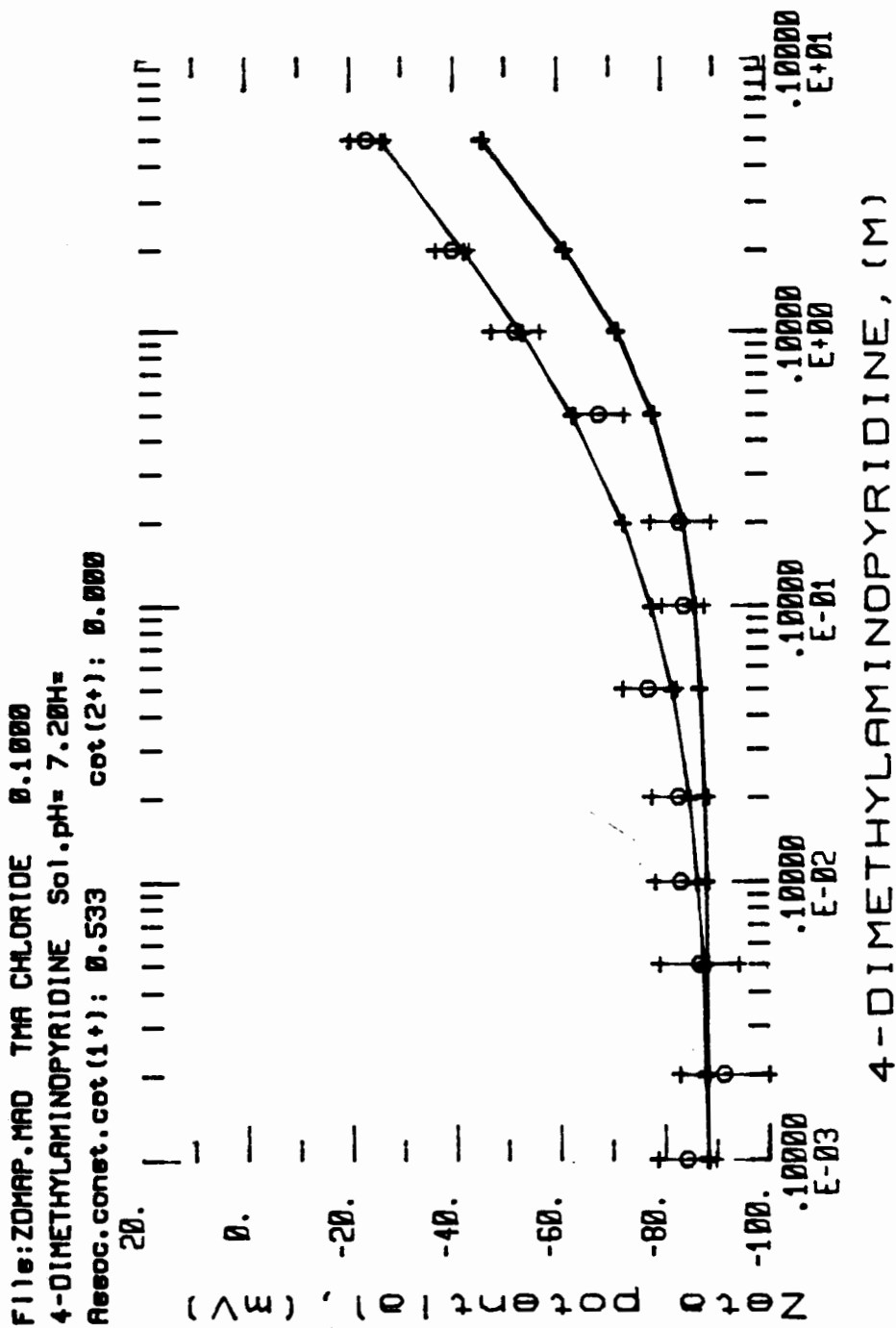


Figure 17. Adsorption isotherm for 4-dimethylaminopyridine.

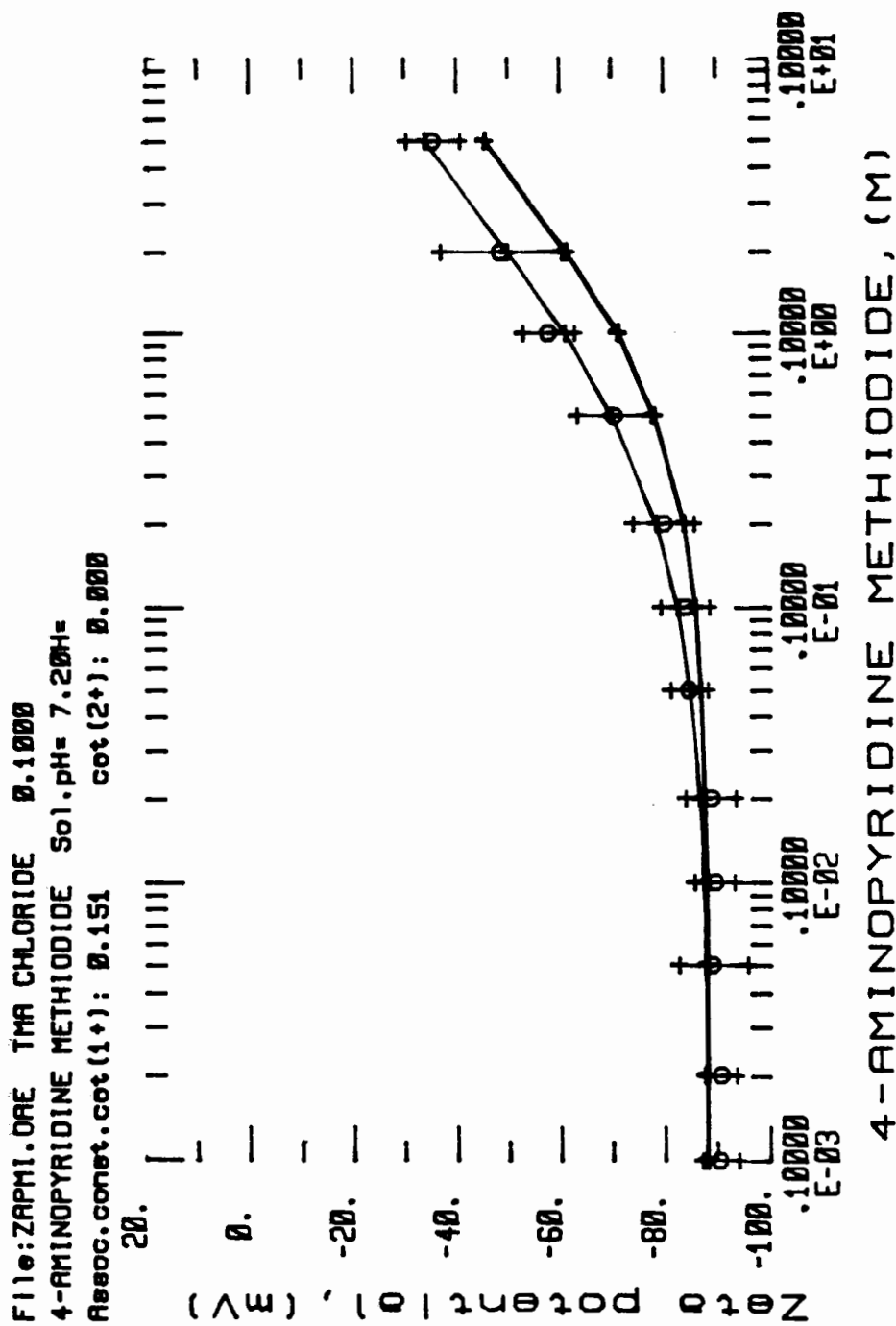


Figure 18. Adsorption isotherm for 4-aminopyridine methiodide.

