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NON-STeady-STATE AND Steady-STATE Membrane Permeation
STUDied BY RotatInG-ELECTRoDE PolaROgraphy

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Yie-wen Chien, B. Sc.

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1972

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INTRODUCTION

Transport processes occurring across a membrane barrier separating a solution phase from another solution phase or a solid phase continue to be of great interest to pharmaceutical scientists and biologists. Pharmaceutical scientists would like to have an understanding of the mechanisms of transport so that, with the knowledge so gained, they would be able to develop new membrane systems having controlled drug-release properties or to use them to design containers to slow drug degradation with little drug-container interaction. Biologists utilize the membranes as simple models for the physiological membranes in order to realize, in terms of well-established physico-chemical principles, the behavior of complex biomembranes and the mechanisms of complicated biological absorption in the body.

There have been numerous studies involving the measurement of mass transport of drug and drug-like molecules across a large variety of membranes or barriers ranging from the non-biological barriers to the biological membranes (1-9). In most instances (1-6, 9), the actual measurement of membrane permeation has been conducted along rather classical lines where the barrier separates two compartments of solu-
tion; the contents of which are sampled and measured as a function of time. Spectrophotometry is the detector most commonly used. The usual time course of measurement of mass transport is, as a result, conducted over a long periods, taking from several hours to over a day, primarily because the ratio of compartment volume to barrier surface-area is relatively large requiring a substantial amount of time before a significant fraction of the penetrating drug species can across the membrane barrier. Furthermore, even in the recently developed systems (7,8), the detector is removed from the immediate surface of the barrier in the receiving compartment resulting in a time lag in transport measurement, even though the sampling procedure is no longer needed.

A review of the current literature generates the observation that a formidable task facing membrane permeation researchers is the design of a suitable diffusional system. Variables such as the integrity, area and thickness of a membrane, volumes and concentrations of donor and receiving compartments, sampling, stirring, as well as temperature must be controlled precisely. These are generally accomplished at the sacrifice of analytical sensitivity. Many cell prototypes may be found in the literature (1-6, 9-16) and, considering that most were designed for similar purposes, their diversity is remarkable. Flynn and Smith (7) have classified these cell systems, based on the gross similarity or dissimilarity of the donor and receiving compart-
ment, size and geometry, as well as membrane placement, and have analyzed their advantages and drawbacks.

The feature of a diffusional system with which a membrane permeation measurement should be most concerned is the ability to reliably detect transported material as early as possible. On the basis of this principle, an effort was made to construct a relatively simple and easy-to-assemble transport cell. Essentially, a membrane sheet is positioned smoothly on the immediate surface of a rotating-disc electrode. Flux is measured polarographically, thus providing a direct measure of rate of transport ranging from non-steady-state through steady-state diffusion. The sensitivity of any diffusion cell will, as pointed out recently by Olson and his associates (8), be proportional to the available diffusional area of a membrane divided by the solution volume of the receiving compartment in which the rate of appearance of penetrant species is being followed. The solution volume of the receiving compartment in our system is made very small. The ratio of diffusional membrane area to compartment volume is therefore extremely high. With the result that the sensitivity of detection is remarkably improved. Moreover, the detector is positioned adjacent to the membrane surface permitting instantaneous measurement of time-dependent diffusion.

Furthermore, the membrane phase is rotated perpendicularly in the donor compartment at a high synchronous rotation
speed, so that the thickness of the hydrodynamic diffusion layer may be controlled and calculated. The use of the rotating disc configuration provides a uniform flux density over the entire surface of the membrane. No other technique provides this advantage.

In our system we combine the fast response of electrochemical instrumentation with the use of a technique that permits consecutive addition of increasing amounts of drug allowing the continuous and simultaneous analysis of both the non-steady-state and steady-state membrane permeation in a single experiment. The high sensitivity, rapid response, and non-sampling method utilized in this system should permit the observation of transient phenomena that may be very significant to the mechanism of membrane transport but which may be overlooked using a slower conventional sampling methodology.

The lag time technique of Daynes (18) and Barrer (19) has been most popularly applied in conventional mass transport systems to calculate the diffusivity of drug species across the barrier phases. It is evident, however, that one should use only those data taken after experimental times larger than about two lag times; otherwise, a significant error will result in the extrapolated lag time and in turn, the calculated diffusivity. Siegel and Coughlin (20) have recently made a theoretical analysis of such a system and concluded that the relative error in lag time can be five
times larger than the error in the slope of a carelessly chosen straight line. It therefore appears that diffusion data obtained from permeation experiments using the time-lag technique based on \( t_{\text{lag}} = \frac{L^2}{6D} \) should be used cautiously. The analysis suggests that the error in diffusivity will always be several times larger than the error in permeability when such technique are employed. A survey of the literature generates the observation that the choice of data must be made judiciously. For instance, a recent report published by Flynn and Roseman (21) in which most of the diffusivity data across dimethylpolysiloxane membranes were calculated from the lag time values extrapolated from the diffusion curves before two lag times, thus it may be possible that an uncertainty is contained in their estimated diffusivity data. Our method allows an estimate of diffusivity from either permeability at steady-state or from a kinetic analysis of non-steady-state transport and not from lag time measurements.

Although extensive experimental and theoretical studies (22) have been carried out on permeation of gases and vapors through various materials, diffusion in polymer membranes has received relatively limited attention; particularly, the dynamic nature of drug permeation across the barriers. In general, two distinctly different phenomenological mechanisms are met in the flow of penetrant species through thin membranes: in one there is molecular transport involving
partitioning and solubilization of permeant in the membrane structure followed by molecular diffusion across it, and in another there is passage of penetrant molecules through membrane pore channels filled with solvent by way of passive molecular diffusion. Transport through the non-porous barriers depends mostly on the relative solubility in the barrier and solubility in the receiving compartment, so the chemical modifications maximizing transport properties must not affect biological activity. This can be a difficult task. On the other hand, the molecular diffusion of drug molecules through the solvent-filled pore channels can be easily controlled at a desired drug-release rate simply by controlling the physico-chemical nature of a polymeric membrane, e.g., selectivity, pore area, porosity, thickness and tortuosity, without sacrifice of the pharmacological activity of the active drug species. The results reported in the present study on the importance of thickness, pore area, porosity and tortuosity of membrane on the membrane permeation may provide some preliminary information with regard to drug-transport modeling. In addition, the successful application of electrochemical instrumentation and consecutive steady-state technique may offer a better methodology for the understanding of membrane permeation mechanisms at both non-steady-state and steady-state transport.
### THEORETICAL BASIS

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A. Diffusion-controlled processes of membrane permeation at non-steady-state and steady-state

Current membrane transport theories may be divided roughly into three groups based on the nature of the flux equation used in its treatment. In the first group fall many of the theories based on the Nernst-Planck flux equations or their refinements. In the second group is included all the theories using the principles of irreversible thermodynamics. The third group involves the theories which utilize principles of the theory of rate processes. In general, the theories of group one involve classical thermodynamics or quasi-thermodynamics which is restricted to isothermal systems. The theories of group two, apart from being more rigorous and realistic, allow a better understanding of transport phenomena in membranes and are useful in dealing with nonisothermal systems. The theories of group three contain parameters which are still unknown for the membrane and hence have limited applicability (23). In view of these three groups of membrane theory, the flux equations grounded on the Nernst-Planck theory were found most useful to the present studies.

Considering a consecutive reaction of the following nature:

\[ \begin{align*}
  & R_x \to 0 \quad \xrightarrow{\text{transport process}} \quad R_x = 0 \quad \xrightarrow{\text{electron-transfer process}} \quad P
\end{align*} \] (1)
Two processes are considered: transport and electron-transfer. The transport of the electron-acceptor, $R$, from the bulk of the solution $x \rightarrow \infty$ to the interface on the electrode surface $x = 0$ precedes the transfer of electrons from the electrode to the electron-acceptor molecules. Finally, the reduced products, $P$, formed at the interface are carried away to the bulk of the solution.

At equilibrium (24), the electron-transfer current density, $i_{ET}$, must be equal to the current density, $i_T$, due to the transport of electron acceptor

$$i_{ET} = i_T = i \quad (2)$$

According to Faraday's law (25), the transport-current, $i_T$, can be defined as

$$i_T = nF \frac{dN}{dt} \quad (3-1)$$

or

$$i_T = nF \cdot J_{\text{transport}} \quad (3-2)$$

where the $dN/dt$ denotes the number of moles of electron acceptor that reach the electrode surface in unit time and subject to electrochemical reduction; $J_{\text{transport}}$ is the flux; $n$ the number of electrons taken up by a single molecule of the electron acceptor during the electron-transfer process; and $F$ the faraday constant.
Combining Eqs. (2) and (3) results in Eq. (4)

\[
\frac{i}{n_F} = \frac{i_{ET}}{n_F} = \frac{i_T}{n_F} = J_{\text{transport}} = \frac{dN}{dt}
\]  

(4)

If diffusion is the transport mechanism, the flux equality condition becomes

\[
\frac{i}{n_F} = \frac{i_{ET}}{n_F} = \frac{i_D}{n_F} = J_D = \frac{dN}{dt}
\]  

(5)

where \( J_D \) is the diffusional flux and \( i_D \) is the diffusion current. Eq. (5) is valid within an infinitesimal time interval \((24), \text{i.e., } dt\).

Based on Nernst-Planck theory, the diffusional flux, \( J_D \), of molecular or ionic species through a membrane under the driving force represented by the chemical potential gradient, \( d\mu /dx \), is given by

\[
J_D = \frac{D}{RT} c \left( -\frac{d\mu}{dx} \right) = -DC \left( \frac{d\ln a}{dx} \right)
\]  

(6)

where \( D \) is the diffusion coefficient; \( a \) is the activity; \( R \) is the gas constant; and \( T \) is the absolute temperature. Substituting \( C r \) for \( a \) (where \( r \) is the activity coefficient) we get

\[
J_D = -DC \left( \frac{d\ln c}{dx} + \frac{d\ln r}{dx} \right) = -D \left( \frac{dc}{dx} + C \frac{dlnr}{dx} \right)
\]  

(7)
When $r$ is constant (ideal case), the flux equation (7) reduces to Fick's law of diffusion:

$$J_D = -D \frac{dC}{dx}$$  \hspace{1cm} (8)

In Eq. (8) $dC/dx$ represents the concentration gradient in one plane across the membrane. This one-dimensional approximation is satisfactory (40) in the present case of membrane transport since the membrane thickness ($\sim 6 \times 10^{-3}$ cm) is much smaller than the membrane surface area (0.54 cm in diameter) available for diffusion.

For the present investigation, in which both the flux and the concentration gradient vary with the time, it is necessary to obtain a solution of the time-dependent diffusion equation.

$$\frac{dC}{dt} = D \frac{d^2C}{dx^2}$$  \hspace{1cm} (9)

which is called Fick's second law of diffusion. The particular solution of Eq. (9) which is applicable to these experiments is specified by the conditions that the entire apparatus is initially devoid of penetrating drug species (at time zero the penetrant is introduced suddenly at a concentration $C_d$ into the donor compartment). The concentration in the receiving compartment remains very small in comparison with the concentration in the donor compartment.
i.e., the concentration of transported drug species at the electrode surface inside the receiving compartment is essentially zero when a constant potential is applied. Consequently, the boundary value of concentration at the electrode surface may be taken as zero.

In the case of diffusion in a single plane through a diffusion path of thickness, $\delta$, Rogers et al. (26) have developed the following equation (10) as the solution to Eq. (9)

$$\frac{dC}{dt} = \frac{ADq}{V \delta} C_d \left[ 1 + \sum_{m=1}^{\infty} 2 \cos m\pi \exp \left( - \frac{m^2 \pi^2 D}{\delta^2} t \right) \right]$$  \hspace{1cm} (10-1)

or

$$\frac{dN}{dt} = \frac{ADq}{\delta} C_d \left[ 1 + \sum_{m=1}^{\infty} 2 \cos m\pi \exp \left( - \frac{m^2 \pi^2 D}{\delta^2} t \right) \right]$$  \hspace{1cm} (10-2)

where $A$ is the cross-sectional area of the diffusion path; $D$ is the diffusion coefficient of the penetrant; $q$ is a constant dealing with the interaction between the penetrant and the membrane; $C_d$ is the initial bulk concentration of penetrant in the donor compartment; $\delta$ is the effective length of the diffusion path; $V$ is the volume of the receiving compartment; and $t$ is the time. Equations similar to Eqs. (10) have been developed by Higuchi and Higuchi (36), Barrer (22), and Autian (58) to express the time-dependent membrane permeation process. Assuming that no interaction occurs between the penetrant and the membrane, i.e., $q = 1$ (For instance, the
transport of penetrant across the porous membrane phase is simply by way of solvent-filled pore channels), and only the early terms are significant to the dynamics of membrane transport, i.e., \( m = 1 \), then \( \cos m \Pi = -1 \) and Eq. (10) may be simplified to

\[
\frac{dN}{dt} = \frac{ADCd}{\delta} \left[ 1 - 2 \exp\left( -\frac{\Pi^2D}{\delta^2} t \right) \right] \tag{11}
\]

Combining Eq. (11) and Eq. (5) gives

\[
i_t = nF \frac{dN}{dt} = nFAD \frac{C_d}{\delta} \left[ 1 - 2 \exp\left( -\frac{\Pi^2D}{\delta^2} t \right) \right] \tag{12}
\]

By inspection of Eq. (12) it can be seen that after a sufficiently long time \( dN/dt \) attains a steady-state value

\[
i_{ss} = nFAD \frac{C_d}{\delta} \tag{13}
\]

where \( i_{ss} \) denotes the diffusion-controlled steady-state current. This equation indicates that electron-transfer flux is limited by diffusion flux at steady-state.

Eq. (12) can be transformed to

\[
i_t = i_{ss} \left[ 1 - 2 \exp\left( -\frac{\Pi^2D}{\delta^2} t \right) \right] \tag{14}
\]

By taking logarithm on both sides of Eq. (14), then

\[
\log\left( i_{ss} - i_t \right) = \log\left( 2i_{ss} \right) - \frac{\Pi^2D}{2.3 \delta^2} \tag{15}
\]
Letting $k_m = \frac{\pi \sqrt{D}}{\delta^2}$ \hspace{1cm} (16-1)

Eq. (15) can be expressed alternatively by

$$\log (i_{ss} - i_t) = \text{Constant} - \frac{k_m}{2.303} t \hspace{1cm} (17)$$

A linear relationship between $\log (i_{ss} - i_t)$ and time should be observed. From the slope of this linear relation, $k_m$ may be estimated. $k_m$ may be defined as the uni-molecular -like rate-constant for membrane transport. It is obvious that Eq. (17) follows the first-order rate law which indicates that the diffusion-controlled membrane transport is mechanistically a unimolecular process.

Also, a linear relationship should be obtained between $i_{ss}$ and $C_d$ at steady-state permeation based on Eq. (13). The slope of this plot is $nFAD/\delta$ which may be defined as the steady-state membrane permeability ($P_m$):

$$P_m = \frac{nFAD}{\delta} = \frac{i_{ss}}{C_d} \hspace{1cm} (18-1)$$

It should be noted that both the rate-constant for membrane transport, $k_m$, and the steady-state membrane permeability, $P_m$, are dependent on the effective length of diffusion path, $\delta$, according to Eq. (16-1) and (18-1). The significance of $\delta$ in the non-steady-state and steady-state membrane permeation will be analyzed later.
B. Time-dependency of diffusion-layer thickness

Considering a stationary planar electrode which is immersed in a solution of an electron acceptor \((R)\); at steady-state, the limiting current produced from the reduction of this electron acceptor is described by Eq. (13-1)

\[
i = nFAD \frac{C_d}{\delta}
\]  

Before the onset of natural convection, i.e., the establishment of a chemical potential gradient resulting from a difference in concentration between electrode surface and bulk solution, the time variation of diffusion-layer thickness depends on whether the diffusion process occurs at a constant potential or a constant current (24). If the diffusion-controlled electrolysis is occurring under a constant potential initiated at \(t = 0\) as in the present transport studies, the diffusion-layer thickness is given by

\[
\delta = \sqrt{\pi Dt}
\]  

Thus, the effective diffusion-layer thickness \((\delta)\) is time dependent and varies with the square-root of time until natural convection sets in and the electron acceptor diffuses from the bulk solution to the immediate surface of the electrode under a chemical potential gradient.
Equation (13-1) may be transformed to

\[ i_Q = nFAD \frac{C_d}{\sqrt{\pi Dt}} \]  

or

\[ i_Q = nFAD^{\frac{1}{2}} \pi^{-\frac{1}{2}} C_d t^{-\frac{1}{2}} \]  

Eq. (20) is very important since it embodies the characteristics of currents controlled by the rate of semi-infinite linear diffusion. For a given electron acceptor species, the plot of \( i_Q \) against \( t^{-\frac{1}{2}} \) should yield a straight line with a slope of \( nFAD^{\frac{1}{2}} \pi^{-\frac{1}{2}} \) at any concentration \( (C_d) \). The treatment of such a simple process of mass transport has to be thoroughly understood before more elaborate problems leading to useful electrochemical approaches can be attacked successfully (27).

Now, if the electrode disc is forced to rotate at a constant speed, a forced convection will result. Under this situation, the convective transport of electron acceptor species to and from the electrode surface is much faster than for natural convection since the concentration gradient extends over a much thinner so-called diffusion layer. The diffusion problems in such a stirred solution can be interpreted, according to Nernst (28) and Brunner (50, 51), with good approximation by assuming the existence of a static diffusion layer of thickness \( (\delta_D) \) on the rotating-disc electrode surface. Furthermore, Levich (52) and Levich (53, 54)
emphasized that a rotating-disc electrode provides especially favorable diffusion conditions since the diffusion layer has the same thickness ($\delta_D$) at every point on the electrode disc when $\delta_D \ll r$ ( $r$ = disc radius ). As long as laminar flow exists at the rotating-disc electrode, Eq. (21) is valid for the diffusion layer thickness. In the present case, the rate of diffusion of the electron acceptor towards the electrode surface can still be expressed by Eq. (13) in the same terms as diffusion in quiet media according to Nernst (28). However, the thickness of diffusion layer is now time independent and can be described by Eq. (21)

$$\delta_D = 1.62 D^\frac{3}{2} \frac{1}{v\delta} w^{-\frac{3}{2}}$$  \hspace{1cm} (21)$$

where $v$ is the kinematic viscosity of the electron acceptor solution and is defined as the viscosity coefficient divided by the density of the liquid ($\frac{\eta}{\rho}$); $w$ is the angular velocity, $2\pi n$, where $n$ is the angular rotation speed (revolution per second) of the rotating-disc electrode. The proportionality factors (55) of the authors cited differ only slightly (1.62; 1.78; 1.75). Here 1.62 is taken from Heyrovsky and Kuta (25).

Substituting Eq. (21) into Eq. (13) gives

$$i_R = 0.62 nFAD^\frac{2}{3} v^{-\frac{1}{6}} w^{\frac{1}{2}} C_d$$

(22)

The effect of angular velocity ($w$) of the rotating-disc electrode on the magnitude of current ($i_R$) is obvious
from Eq. (23)

\[ \frac{i_R}{C_d} = \left( 0.62 \, \text{nFAD}^{\frac{2}{3}} \, v^{\frac{1}{6}} \right) \, w^{\frac{1}{2}} \]  

(23)

where \( \frac{i_R}{C_d} \) denotes the current per unit concentration of electron acceptor at steady-state. A linear relationship should be observed between \( \frac{i_R}{C_d} \) and \( w^{\frac{1}{2}} \) and its slope should be \( 0.62 \, \text{nFAD}^{\frac{2}{3}} \, v^{\frac{1}{6}} \) which is constant for a given species of electron acceptor in a given supporting electrolyte system. For example, theoretical value of \( 0.62 \, \text{nFAD}^{\frac{2}{3}} \, v^{\frac{1}{6}} \) for p-Nitrophenol in pH 4.0 Acetate buffer at 30°C is \( 9.98 \times 10^3 \) μA·sec\(^{\frac{3}{2}}\)/M.

When a membrane is covering the immediate surface of electrode disc as a barrier to the diffusion of drug, the theoretical model of linear diffusion expressed by Eq. (20-2) should also be obeyed if the membrane structure is inert in nature and acts simply to decrease the diffusional area available for drug transport. If this is the case, then the current flux defined in Eq. (20-2) should be proportionately decreased by a factor equivalent to the membrane porosity (\( \varepsilon \)) since the diffusional area term, \( A \), now is \( A \varepsilon \),

\[ i_m = nF \varepsilon D^{\frac{1}{2}} \Pi^{-\frac{1}{2}} C_d \, t^{-\frac{1}{2}} \]  

(25)

Theoretically, a linear relationship with a slope of \( nF \varepsilon D^{\frac{1}{2}} \Pi^{-\frac{1}{2}} C_d \) and an intercept at origin should be observed between the current height with covering membrane, \( i_m \), and
the reciprocal of square-root of time, \( t^{-\frac{1}{2}} \). The theoretical slope of this linear plot will be equal to the theoretical slope of Eq. (20-2) multiplied by membrane porosity \( (\varepsilon) \), i.e., \( nFAD^\frac{1}{6} \varepsilon^{-\frac{1}{3}} \cd \).

C. Membrane thickness and membrane permeation

After extensive studies on membrane permeation, Lakshminarayanaiah (56) came to the conclusion that all flows were composed of two components: diffusional and viscous. The proportion of diffusional flow in the total flow increases with increasing thickness of the membrane. The importance of membrane thickness in relation to membrane permeation has been well-established in theory (26, 36, 22, 41). Experimentally, it has also been shown that the diffusion of a permeant species through a polymeric membrane is inversely proportional to thickness raised to a constant exponent (21, 41-43).

In the present system, steady-state permeability \( (P_m) \) can be derived from Nernst's diffusion model, i.e., Eq.(13) defined previously, as follows:

\[
P_m = \frac{\text{i ss}}{\cd} = nFAD \frac{1}{\delta}
\]

(18-1)

Thus, the steady-state permeability is proportional to the reciprocal of the effective thickness of the diffusion path \( (\delta) \). For a naked electrode, i.e., no covering membrane,
this effective thickness is equal to the thickness of diffusion layer, \( \delta_D \), at the electrode surface; and for an electrode covered with a membrane of thickness \( \delta_m \), the thickness corresponds to the sum of the thickness of diffusion layer and of the membrane, \((\delta_D + \delta_m)\). If the membrane framework acts only to lengthen the path of the diffusing species (32-35), then

\[
P_m = \frac{n \text{FAD}}{\delta_D + \delta_m} \tag{18-2}
\]

A plot of \( P_m \) against \( 1/(\delta_D + \delta_m) \) should yield a straight line with a theoretical slope of \( n \text{FAD} \).

Eq. (18-2) in its logarithmic form is

\[
\log P_m = \log n \text{FAD} - \log (\delta_D + \delta_m) \tag{26}
\]

showing that plots of \( \log P_m \) versus \( \log (\delta_D + \delta_m) \) should have a slope of -1 in all the membrane permeation systems.

Similar arguments can also be applied to the kinetic aspect of membrane permeation. Thus, Eq. (16-1) can be transformed to

\[
k_m = \frac{n^2 \text{D}}{(\delta_D + \delta_m)^2} \tag{16-2}
\]

where the rate-constant for membrane transport, \( k_m \), is inversely proportional to the square of effective length, \((\delta_D + \delta_m)^2\). Thus, a plot of \( k_m \) versus \( 1/(\delta_D + \delta_m)^2 \) should
yield a straight line with a theoretical slope of $\tau^2 \Delta D$.

Taking the logarithm of both sides of Equation (16-2)

$$\log k_m = \log \tau^2 D - 2 \log (\delta_D + \delta_m) \quad (27)$$

shows that a plot of $\log k_m$ against $\log (\delta_D + \delta_m)$ should give a straight line with a slope of -2.

D. Membrane pore size and membrane permeation

Theoretical studies made by DeBoer (29) on the mobility of materials through pores showed that transport in macro-pores (> 500 Å) is governed by normal diffusion. The pore size of Nuclepore and Millipore membranes investigated in the present study is in the range of macropores, so the permeation across these two types of membranes should also follow the model of normal diffusion; this means the diffusion through the solvent-filled pore channels in these membrane structures should obey the same laws of diffusion (Fick's laws) as those in the bulk solution.

Permeation through a porous membrane depends on many variables. One of these is the pore size (Å). If a round pore and a spherical penetrant species are assumed, the penetrating species will have little difficulty entering the pore if it is very much smaller than the pore. The probability of entry, however, becomes rapidly smaller as the diameter of the sphere approaches that of the pore. For the large pore case practically all of the cross-sectional area
of the pore is available for diffusion, but in the small pore situation only the difference between pore area and the cross-sectional area of the penetrant sphere (45-49) is available. Uzelac and Cussler (30) have related their observed variation of the diffusion coefficient across Millipores with the pore diameter. Furthermore, Ullah and Cadwallader (31) report a direct relationship between the time needed for establishment of equilibrium diffusion and the pore size of screens and membranes.

In a series of publications, Manegold (32-35) elaborated a quantitative theory relating the rate of diffusion in a liquid-filled network to the average pore size of six types of membrane pores. He came to the conclusion that at a sufficiently large pore size macromolecular ions and molecules migrate in the pore channels with the same speed as in the bulk of the solution phase. He found that if a membrane is inert physico-chemically, it acts only by diminishing the effective area across which diffusion occurs and by lengthening the path of the diffusing drug species.

On the basis of the literature (29-35) discussed above, it is obvious that both the steady-state membrane permeability \( (P_m) \) and the rate-constant for membrane transport \( (k_m) \) should be sensitive to the variation in size of the membrane pores at and only when the pore size approaches the size of the penetrant molecule, if there are no other variables such as tortuosity.
E. Membrane tortuosity and membrane permeation

Higuchi and Higuchi (36) have made a theoretical analysis of diffusional movement through heterogeneous barriers and observed that both the steady-state diffusion and non-steady-state transport would be inversely proportional to the tortuosity, $\mathcal{J}$, of the barriers. Tortuosity is defined (21) as

$$\mathcal{J} = \frac{\delta^*_m}{\delta_m} \quad (28)$$

where $\delta^*_m$ is used to denote the average effective diffusional pathlength across the barrier and $\delta_m$ is the overall thickness of the barrier. Flynn and Roseman (21) have recently reported that the lag time for membrane transport is dependent on the tortuosity squared since the thickness term in the Barrer equation for lag time is replaced by the effective diffusional pathlength defined in Eq. (28) here.

Also, Farhadieh et al. (37) have found that the drug-release rate from the tablets made with a methylacrylate-methyl-methacrylate copolymer containing dispersed solid drug is decreased by exposure to acetone vapor. The reduction in the drug-release rate constant was assumed to be primarily due to increased tortuosity of the polymer film upon acetone vapor exposure. Tortuosity values can range from 1 to 200.
By referring to Eq. (28), Eq. (18-2) and (16-2) may be transformed to

\[ P_m = \frac{nFAD}{(\delta_D + \delta_m^*)} \quad (29-1) \]

or

\[ P_m = \frac{nFAD}{(\delta_D + \mathcal{T} \cdot \delta_m)} \quad (29-2) \]

and

\[ k_m = \frac{\Pi^{2D}}{(\delta_D + \delta_m^*)^2} \quad (30-1) \]

or

\[ k_m = \frac{\Pi^{2D}}{(\delta_D + \mathcal{T} \cdot \delta_m)^2} \quad (30-2) \]

It is apparent that the higher the membrane tortuosity, the lower will be the values of \( P_m \) and \( k_m \). The effect of tortuosity on the dynamics of membrane transport is obviously greater than that on the permeation at steady-state (compare Eq. 30 with Eq. 29).
Diffusion through membranes is fundamentally no different from diffusion in liquid systems. In theory Fick's laws of diffusion apply to both. The mechanism involves transfer of a molecule to a "hole" in the medium. Energy of activation is required for formation of such a hole whether in a liquid medium or in a membrane structure (57). In other words, diffusion is a process in which molecules of a liquid or solute disperse or intermingle, by virtue of their thermal energy, against a drag or resistance due to the viscosity of the medium. It is a spontaneous process which leads ultimately to thermodynamic equilibrium and is accompanied, when conducted isothermally as in the present studies, by a decrease in total free energy (57).

The temperature-dependency of diffusivity (D) has been well-established theoretically and experimentally (38-40, 57, 58). It takes the form:

\[ D = D_0 \exp \left( - \frac{\Delta E_D}{RT} \right) \]

where \(D_0\) is a pre-exponential factor, \(\Delta E_D\) the activation energy for diffusion, \(R\) the ideal gas constant, and \(T\) the absolute temperature.

Both the steady-state membrane permeability \((P_m)\) and the rate-constant for membrane transport \((k_m)\) are directly proportional to the diffusivity \((D)\) as defined by Equations (29)
and (30); therefore, the temperature-dependency of \( k_m \) and \( P_m \) can be derived as follows:

\[
P_m = \frac{nFA}{(\delta_D + J \cdot \delta_m)} D \quad (29)
\]

\[
k_m = \frac{\Pi^2}{(\delta_D + J \cdot \delta_m)^2} D \quad (30)
\]

In a controlled system with the various parameters kept constant:

\[
P_m \times \frac{(\delta_D + J \cdot \delta_m)}{nFA} = D = D_o \exp \left( -\frac{\Delta E_D}{RT} \right) \quad (31)
\]

\[
k_m \times \frac{(\delta_D + J \cdot \delta_m)^2}{\Pi^2} = D = D_o \exp \left( -\frac{\Delta E_D}{RT} \right) \quad (32)
\]

Letting \( P_m^o = D_o \frac{nFA}{(\delta_D + J \cdot \delta_m)} \) \( (33) \)

\[
k_m^o = D_o \frac{\Pi^2}{(\delta_D + J \cdot \delta_m)^2} \quad (34)
\]

the following equations will result:

\[
P_m = P_m^o \cdot \exp \left( -\frac{\Delta E_p}{RT} \right) \quad (35)
\]

\[
k_m = k_m^o \cdot \exp \left( -\frac{\Delta E_k}{RT} \right) \quad (36)
\]

where \( P_m^o \) and \( k_m^o \) also denote pre-exponential factors; \( \Delta E_p \) and \( \Delta E_k \) are essentially the same as \( \Delta E_D \) and defined as the acti-
vation energies for permeability and for transport respectively.

Taking the logarithm of both sides of Equations (35) and (36) gives

\[
\log P_m = \log P_m^0 - \frac{\Delta E_p}{2.303 R T} \frac{1}{T} \quad (37)
\]

\[
\log k_m = \log k_m^0 - \frac{\Delta E_k}{2.303 R T} \frac{1}{T} \quad (38)
\]

Equations (37) and (38) demonstrate that a linear relationship exists between \(\log P_m\) and \(1/T\) and between \(\log k_m\) and \(1/T\). From the slope of these straight lines, the activation energies for membrane permeation (\(\Delta E_p\)) and for membrane transport (\(\Delta E_k\)) can be determined.
## METHODOLOGY

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<td>63</td>
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A. **Electrochemical system.**

The experimental system employed in these studies is schematically presented in Figure 1. All transport processes were studied in a specially fabricated H-type polarographic cell which was immersed in a water-bath thermostated with a Sargent Thermonitor model ST. The lefthand half-cell is a Saturated Calomel Electrode (S.C.E.) as the reference electrode (60) which is electrically connected to the righthand half-cell, the working compartment, via a saturated KCl salt bridge. The working compartment contains a small volume (10-20 ml) of test solution into which the working electrode is immersed and rotated at a given constant speed. Both the working electrode and the reference electrode are electrically connected via a polarograph module.

A well-balanced Heath-Built model EUA-19-4 works with a Heath-Built Polarography Module model EUA-19-2 to function as a command center to supply an applied potential across the electrodes and also to amplify the resultant current from the oxidation or reduction of a given electroactive drug species. This amplified current may be recorded as function of applied potential or time on the chart of a calibrated Varian Associates model G-14 Automatic Recorder or on the screen of Hewlett Packard 141A Oscilloscope triggered by a Wavetek Trigger VCG model 112. The use of either the recorder or the Oscilloscope is decided by the
Figure 1  Schematic representation of the electrochemical system used to measure drug transport.
time scale required to trace the course of transport across the barrier phase. For time scales longer than several tenths of a second, the recorder is found to be useful, and for time scales down to milli- or micro-second, the Oscilloscope is used. The current-time curve generated on the screen of the Oscilloscope may be photographed.

The test solution in the right polarographic cell (working compartment) is deaerated with a stream of pure nitrogen gas just before the measurement. During the course of experiment, a nitrogen atmosphere can be maintained on the surface of the test solution to prevent it from contamination by an undesired gas (e.g., oxygen should be absent for the reduction of nitrophenols).

The drug concentration in the test solution may be changed as desired by injecting a small amount (0.2-1.0 ml) of a predeaerated concentrated drug solution into the test solution via the drug inlet. This technique, referred to as the "Consecutive Steady-state Technique", has been found to be very satisfactory and useful in the study of both steady-state permeability and non-steady-state membrane transport kinetics.
B. Synchronous rotating-disc electrode as a working electrode.

For the purpose of studying the dynamics of mass transport of drugs across a membrane, a membrane sheet is positioned on the immediate surface of an electrode (Figure 2) with the aid of a specially designed transport cell made from Teflon. A drop of test solution is delivered to the cell opening to cover the positioned membrane after assembly and caution is taken not allow the presence of bubbles in the cell opening. This combination is rotated at a high synchronous speed in the test solution containing the electroactive drug species. Under this condition the thickness of the diffusion layer on the membrane surface may be decreased to a point at which the thickness of the hydrodynamic diffusion layer is constant and usually small in comparison with that of the membrane phase studied; therefore, transport-through-the membrane becomes the rate-limiting step in the mass transport process.

The synchronous rotating-disc electrode first used was the Sargent Synchronous Rotator which gives a constant rotation speed of 600 r.p.m.. A mercury-coated platinum disc electrode can be made (59) routinely as needed and was used in the preliminary studies. This single speed rotator was replaced by a Beckman Rotating Electrode Assembly (Figures 1 & 2) in the later transport studies.
Figure 2 Schematic representation of the membrane holder (Transport cell) assembly showing its relation to the electrode tip. The electrode tip can be removed from the rotator. The cap is placed over the electrode tip with a membrane attached and the body is then attached.
The Beckman Rotating Electrode Assembly consists of a driving unit (188501 w), a rotating electrode body (188551 w), and an interchangeable electrode disc (metal or carbon core). The driving unit consists of a synchronous motor, a push-button power switch and a speed control (0.6 to 6000 revolution per minute). A flexible shaft connects the motor to the rotating electrode body and then to the electrode disc. A carbon contact completes the electric circuit from the rotating electrode disc to the measuring device in the polarograph module mentioned previously (Figure 1).

There are two basic reasons for using a rotating-disc electrode in preference to a conventional dropping mercury electrode (D.M.E.): namely, increased signal and greater usable potential range. The increase in signal results from the decrease in the thickness of the hydrodynamic diffusion layer with the resultant enhancement of mass transport. This increase of signal-to-background may provide an order of magnitude better detectability than a DME. A typical comparison is shown in Table 1 where current flux is increased 50-fold by the use of a rotating-disc electrode when compared with a dropping mercury electrode.

Two types of electrode disc were used in these studies: (i) A Beckman platinum electrode (39086) can be electrochemically coated with a thin film of mercury based on the method reported by Rameley et al. (59). This mercury-coated platinum electrode combines the advantages of the electro-
<table>
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<tr>
<th>Electrodes</th>
<th>(i_{\text{SS/conc.}}(\times 10^4 \mu A/M))</th>
<th>(E_d) (Volts)</th>
<th>(E_{1/2}) (Volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beckman Platinum(^a) (Hg-coated)</td>
<td>61.5</td>
<td>- 0.48</td>
<td>- 0.63</td>
</tr>
<tr>
<td>Dropping Mercury(^b)</td>
<td>1.26</td>
<td>- 0.52</td>
<td>- 0.68</td>
</tr>
</tbody>
</table>

\(^a\) Rotation speed: 3000 rpm.

\(^b\) From Reference (66).

\(^c\) 1x10\(^{-4}\) M of p-Nitrophenol at pH 4.0 and 30\(^\circ\)C.
chemical properties of both platinum and mercury. The mercury-coated platinum electrode shows the same electrochemical properties as the dropping mercury electrode as demonstrated experimentally in Table 1. (ii) A Beckman carbon electrode tip (39084) was used in later experiments on the basis of four advantages it offers: (a) wide range of applied potential in both cathodic and anodic regions; (b) low residual currents; (c) greater reproducibility than noble metal electrodes, e.g., platinum; and (d) little or no pretreatment is necessary. Thus, the carbon electrode in some instances is more useful than the platinum electrode. Table 2 shows the difference in electrochemical character between a mercury-coated platinum electrode and a carbon electrode.

Both these electrodes have a smooth and flat electrode surface. This adds an additional advantage to the membrane permeation studies since the smooth and flat electrode surface allows the tested membrane sheet to be positioned evenly on the immediate surface of the rotating-disc electrode without changing the physical texture of the membrane. The entire membrane surface is in contact with the electrode.
### TABLE 2

THE ELECTROCHEMICAL PROPERTIES OF VARIOUS NITROPHENOLS AT THE CARBON AND MERCURY-COATED PLATINUM ELECTRODES*

<table>
<thead>
<tr>
<th>Nitrophenols</th>
<th>Concentration (x 10^5 M)</th>
<th>E_d (Volts)</th>
<th>E_k (Volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hg</td>
<td>Carbon</td>
</tr>
<tr>
<td>p -</td>
<td>4</td>
<td>-0.48</td>
<td>-0.70</td>
</tr>
<tr>
<td>o -</td>
<td>5</td>
<td>-0.34</td>
<td>-0.56</td>
</tr>
<tr>
<td>m -</td>
<td>5</td>
<td>-0.33</td>
<td>-0.55</td>
</tr>
</tbody>
</table>

* Both of these electrodes were purchased from Beckman Instruments Inc. and rotated at 3000 rpm, pH 4.0, 30°C. The platinum electrode was coated with a mercury film by the method described in Reference (59).
C. Transport cell

A small, light-weight, and easy-to-assemble transport cell made from Teflon was specially designed to hold a thin membrane sheet on the flat surface of the electrode disc. One of its advantages is that the solution volume of the receiving compartment is reduced to a small value; essentially no spacing exists between the immediate surface of electrode and the inner surface of membrane barrier as evident from experimental observations. In this way, the drug species may be oxidized or reduced by the electrode on the immediate inside surface of the barrier phase, thus cell sensitivity was expected to be very high and the time required to reach steady-state flux should be much shortened on the basis of the arguments by Olson et al. (8).

A tiny hole is drilled on the side of the upper portion of the transport cell (Figure 2) for the purpose of balancing the pressures inside and outside the cell to prevent the possible effect of hydrodynamic pressure.

The transport cell designed may be assembled just before measurements are made and easily separated for cleaning. Through the use of this cell, a membrane sheet can be easily mounted directly on the immediate surface of electrode smoothly without sacrificing the original texture of the membrane (in Figure 2, actually no spacing is allowed between the surfaces of electrode and membrane).
D. Buffered supporting electrolyte system.

In the absence of a supporting electrolyte, there would be an electrostatic force acting on the electroactive ions. This would result from the gradient of electrical potential between the working and the reference electrodes in the polarographic cell, which caused ions to move through the solution in order to carry the current through the cell. The migration of ions under the action of this electrical potential gradient will result in a migration current which may complicate the diffusion current resulting from the diffusion-controlled mass transport process. Because of this migration, the number of electroactive ions reaching the electrode surface in a unit time is greater than it would be under the influence of diffusion alone. Thus, this acting force tends to enhance the diffusion of the electroactive ions, the current due to the electrolysis of the ions is greater than the diffusion current. On the other hand, the current due to the electrolysis of an electrolyzable ion is less than the diffusion current if this acting force tends to retard the diffusion of the electroactive ions. Therefore, supporting electrolyte must be included in the test solution to eliminate the effect of migration current and thus allowing a measurement of diffusion current. In addition, a high concentration of supporting electrolyte is utilized to reduce the IR drop through the polarographic cell to a negli-
gible value as well as to buffer the solution of dissociating substances. In this way, a true diffusion-controlled current can be observed (61).

Hydrogen ions are consumed in the reduction (and liberated in the oxidation) of most organic compounds. The variation of pH at the immediate surface of electrode sometimes causes pronounced changes in the characteristics of the polarogram; therefore, it is wise to buffer the supporting electrolyte solution and the test drug solution to avoid this complication (60-62).

Any electrolyte, which does not interfere with the electrode reaction, can be employed as the supporting electrolyte for the drug solutions tested. As a general rule, the molar concentration of the least concentrated component of the buffer system should be at least 20P times that of the drug species being studied, where P is the number of hydrogen ions consumed or liberated in the reduction or oxidation of each molecule of the drug species. Thus, the supporting electrolyte will almost entirely carry the current in the polarographic cell completely eliminating the effect of migration. Experimentally, there results a well-defined residual current ($i_r$) as shown in Figure 3.

The following two supporting electrolyte systems have been found experimentally to be useful in these studies:
Figure 3 The current-potential curve for the electroreduction of Nitrophenol isomers.

\[ i_p \] = the current height at plateau
\[ i_r \] = the residual current from supporting electrolyte system
\[ i_{ss} \] = the diffusion-controlled steady-state current from Nitrophenol isomers
\[ E_{1/2} \] = the half-wave potential
\[ E_d \] = the potential of decomposition
(1) Acetate Buffer* (pH 4.0)

Potassium Acetate 0.2 mole
Glacial Acetic Acid q.s. to get pH 4.0
Double Distilled Water q.s. to make 1000 ml.

(2) Phosphate Buffer* (pH 7.4)

K$_2$HPO$_4$ (or Na$_2$HPO$_4$) 0.08 mole
KH$_2$PO$_4$ (or NaH$_2$PO$_4$) 0.02 mole
Double Distilled Water q.s. to make 1000 ml.

* All the reagents are "Baker Analyzed" reagent grade and purchased from J. T. Baker Chemical Co. in Phillipsburg, N. J.

In these two buffer systems, the potassium salt (or sodium salt) functions as the supporting electrolyte. Theoretically, potassium ion has a high negative half-wave potential (E$_{\frac{1}{2}}$) around -2.0 volts. Experimentally, it is observed that potassium ion yields a low residual current before -1.2 volts and is therefore useful for the studies on the reduction of aromatic Nitro compounds, e.g., Nitrophenols (Figure 3).
E. Model drugs for membrane permeation studies.

Nitrophenol isomers were chosen as the model drugs for the studies of the mechanism of membrane transport on the basis of the following criteria:

(1) The cross-sectional area of nitrophenol molecules is much smaller than the single pore area of the membrane with the smallest pore size, i.e., there is at least 2500-fold difference. Thus, the transport of nitrophenol molecules across the membrane should not be complicated by any effect of molecular sieving.

(2) The relative spatial position of the electron-attracting nitro group to the electron-donating phenolic hydroxyl group on the aromatic ring system is different in these three nitrophenol isomers. This difference will result in different physico-chemical properties (Table 3) which may be involved in the intramolecular or intermolecular interactions of one nitrophenol molecule with another, with other chemical species coexisting in the same solution, and/or with the membrane phase.

(3) The electroreduction behavior of Nitrophenols has been extensively studied using D.M.E. Polarography (63, 66).

These nitrophenols were purchased from Eastman Organic Chemical Co.. Their purity has been checked by both the pH-
<table>
<thead>
<tr>
<th>Physico-chemical Properties</th>
<th>para-</th>
<th>ortho-</th>
<th>meta-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion Coefficient</td>
<td>9.18</td>
<td>7.76</td>
<td>6.34</td>
</tr>
<tr>
<td>( \times 10^{-10} \text{ cm}^2/\text{sec.} ) (^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular Surface Area</td>
<td>25.4</td>
<td>35.6</td>
<td>53.3</td>
</tr>
<tr>
<td>( \times 10^{-16} \text{ cm}^2 ) ( ^b )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK(_a) Values</td>
<td>7.15</td>
<td>7.17</td>
<td>8.28</td>
</tr>
<tr>
<td>(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipolar Moment and its Vector (^d)</td>
<td>2.4D</td>
<td>6.5D</td>
<td>11.2D</td>
</tr>
<tr>
<td>( \text{E}_1 ) (volts) in 10 % Ethanolic solution (pH 4.0)(^a)</td>
<td>-0.470</td>
<td>-0.365</td>
<td>-0.355</td>
</tr>
<tr>
<td>Melting Point</td>
<td>114</td>
<td>45</td>
<td>97</td>
</tr>
<tr>
<td>(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Reference (63)  
\(^b\) Reference (64)  
\(^c\) Reference (65)  
\(^d\) Reference (67) and (68)
titration method (69) and polarographic identification (63) and found to be pure. Their pK values and Electrochemical character (Tables 2, 3 & 4) are very close to that reported in literature (65, 66).

The nitrophenol solution of a given concentration is prepared simply by dissolving an accurately weighed amount of the compound in an appropriately buffered supporting electrolyte system. The pH of a final solution was checked and no change in pH value was observed upon dissolution.

The electroreduction and membrane permeation character of nitroanilines and nitrobenzoic acids were also studied for comparison. They were also purchased from Eastman Organic Chemical Co. and used without further purification.
TABLE 4

pKa AND PURITY OF NITROPHENOL ISOMERS USED IN THE TRANSPORT STUDIES

<table>
<thead>
<tr>
<th>Nitrophenol</th>
<th>Puritya (%)</th>
<th>pKₐ Values</th>
<th>Literature</th>
<th>Experimentala</th>
</tr>
</thead>
<tbody>
<tr>
<td>para -</td>
<td>98.9</td>
<td>7.15</td>
<td>7.12</td>
<td></td>
</tr>
<tr>
<td>ortho -</td>
<td>99.8</td>
<td>7.17</td>
<td>7.26</td>
<td></td>
</tr>
<tr>
<td>meta -</td>
<td>99.84</td>
<td>8.28</td>
<td>8.34</td>
<td></td>
</tr>
</tbody>
</table>

a Determined by titrating 20 mg/100 ml of Nitrophenol with 0.01010 N Standardized NaOH Solution.

b Reference (65).
F. Deaeration of the test solution

Oxygen is reduced at an electrode in two steps involving the formation of \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O} \), respectively. The corresponding half-wave potentials \( (E_\frac{1}{2}) \) are approximately \(-0.1\) and \(-1.0\) volt (vs. S.C.E.), and consequently the two oxygen waves cover a major fraction of the potential range which is to be explored. Air-saturated solutions contain about \( 2.5 \times 10^{-4} \) M of oxygen at room temperature. This is precisely the order of magnitude of the concentration most frequently determined by polarography, and the removal of oxygen is thus essential (60-62).

Since the solubility of oxygen is proportional to the partial pressure of this gas above the solution (Henry's law), oxygen may be removed from solution by bubbling pure nitrogen gas through the solution. The rate of removal of oxygen increases with the area of contact between the nitrogen gaseous phase and the solution phase. For this purpose, gas dispersing disks are incorporated into the righthand half-cell of polarographic cell to effectively deaerate the test solution and provide \( \text{N}_2 \) gas atmosphere (Figure 1). A deaeration column (Figure 4) was also specially constructed for the deaeration of protein solutions or of concentrated drug solutions used in the consecutive addition technique. In the preliminary studies, several experiments were carried out to check the effectiveness of deaeration. Deaeration time of 5 minutes was found to be satisfactory.
Figure 4. Deaeration column for concentrated drug solution or protein-containing solution.
G. Electrochemical instrumentation.

When a test solution containing a given electrochemically oxidizable or reducible chemical species is oxidized or reduced in a H-type polarographic cell under a given applied potential, a stream of current will flow between the working and the reference electrodes. As a representative example, the reduction of aromatic nitro compounds is shown in Figure 5.

By changing the potential difference between the working electrode and the reference electrode, a characteristic current-potential curve is observed (Figure 3). In this curve are shown the half-wave potential \( E^{1/2}_{\text{a}} \) which is characteristic of a given chemical species and the limiting current at plateau \( (i_p) \). The diffusion current at steady-state \( (i_{ss}) \), which is directly proportional to the concentration of this electroactive drug species in the donor compartment, may be calculated as follows:

\[
i_{ss} = i_p - i_r
\]  

(39)

If we apply a constant potential, which is higher than the half-wave potential and in the neighborhood of plateau potential \( (E_p) \) range, a steady-state current, \( i^*_{ss} \), will be attained soon after the start of measurement. The diffusion current at steady-state may be estimated from the following equation:

\[
i_{ss} = i^*_{ss} - i_r
\]  

(40)
Figure 5  Electrochemical reduction pathway of Aromatic Nitro compounds used in the membrane transport studies.
The same steady-state diffusion current \(i_{ss}\) will be obtained from both the current-potential curve and the current-time profile, that is, the values of \(i_{ss}\) calculated from Eq. (39) or from Eq. (40) should be the same when the same potential is chosen. As demonstrated in Nernst equation (Eq. 13) discussed earlier, the steady-state diffusion current \(i_{ss}\) should be linearly related to the bulk concentration of a given electroactive drug species in the donor solution \(C_d\):

\[
i_{ss} = \frac{nFAD}{\delta} C_d \quad (13)
\]

For a rotating-disc electrode, since the thickness of hydrodynamic diffusion layer \(\delta_d\) is a constant term and defined by \(\delta_d = 1.62 D^{\frac{1}{3}} v^{\frac{1}{6}} w^{\frac{1}{2}}\), the steady-state diffusion current can also be expressed by Eq. (22) (developed previously),

\[
i_{ss} = 0.62 nFAD^{\frac{2}{3}} v^{\frac{1}{6}} w^{\frac{1}{2}} C_d \quad (22)
\]

For a controlled system

\[
i_{ss} = P_m \cdot C_d \quad (13-1)
\]

where the steady-state permeability \(P_m\) may be expressed alternatively (in addition to Eq. (18-1) developed previously) by the following Eq. (41):
The linear relationship between \( i_{ss} \) and \( C_d \) as predicted by Eqs. (22) and (13-1) is illustrated in Figure 6 for the case of a naked electrode, i.e., no membrane is covering its surface.

\[
P_m = 0.62 \text{nFAD}^\frac{2}{3} \frac{V}{\varepsilon} \frac{1}{w^2}
\]
Figure 6  Linear relationship between steady-state current ($i_{ss}$) from the reduction of p-Nitrophenol at a naked electrode and its molar concentration ($C_d$) in the bulk solution.

$P_m = 7.82 \times 10^5 \mu A/M$
H. Technology of measurement.

Several techniques have been developed and tested during the course of preliminary studies in order to determine the best methodology in the study of membrane transport mechanisms. The following "Consecutive Steady-state Technique" has been found to be the best method, since it allows the simultaneous measurement of both the non-steady-state kinetics of membrane transport and the steady-state membrane permeability. Its procedure can be briefly summarized as follows:

1. A drug solution of appropriate concentration is prepared in a supporting electrolyte system buffered to a desired pH and ionic strength. It is deaerated in the deaeration column (Figure 4) with a pure nitrogen gas stream (40 mm) for 3 minutes.

2. 17 ml of the same supporting electrolyte solution (free of drug) is delivered into the righthand half-cell of the polarographic cell and deaerated with another stream of nitrogen gas (50 mm) for 5 minutes (Figure 1). After deaeration, a nitrogen atmosphere (35 mm) is maintained above the surface of the solution phase.

3. The transport cell is assembled on the electrode disc with or without a sheet of membrane and then the whole transport cell-membrane-electrode com-
bination is mounted on the rotating electrode body (Figures 1 & 2) and rotated at a given synchronous speed in the solution phase for a given period of time to equilibrate the phases of solution, membrane and transport cell. Consideration is also given to the positioning of transport cell, the nitrogen pressure on the solution surface and the temperature.

(4) A constant potential of -1.0 volt is applied over the working and reference electrodes. A steady-state residual current \( i_r \) is attained in a short time (Figure 7).

(5) 1.0 ml of drug solution (well-deaerated as described in step (1)) is anaerobically transferred with a calibrated microsyringe to the solution phase via the drug inlet (Figure 1). After a short induction time, the current height, which is proportional to the diffusional flux of drug molecules as described in Eq. (5), increases as a function of the time until the time-independent steady-state current height is obtained.

(6) Another 0.4 ml of the same drug solution may be transferred into the solution phase to produce another higher steady-state current. This process may be repeated as many times as desired to obtain
Figure 7 Relation between current ($i$) and time in the transport of p-Nitrophenol through a Nuclepore membrane ($41.6 \times 10^{-4}$ cm thick, 2.0 microns pore) where successive amounts of compound are added.
several steady-state current data.

(7) From this current-time curve, two types of information may be obtained: Non-steady-state kinetics of membrane transport and steady-state membrane permeability can be determined by respectively plotting \( \log (i_{ss} - i_t) \) versus time based on Eq. (17) or by plotting \( i_{ss} \) against corresponding drug concentration in the donor solution based on Eq. (13). Typical examples are shown in Figures 8 & 9 respectively.

In addition, a more conventional technique called here "Single Steady-state Technique" was also used. Its procedure can be briefly described as follows:

(1) A series of drug solutions of increasing concentration are prepared in a supporting electrolyte buffered to a desired pH and ionic strength. Delivery of 20 ml from one of these solutions is made into the working compartment of the polarographic cell and deaerated with a stream of \( \text{N}_2 \) (50 mm) for 5 minutes. After deaeration, a nitrogen atmosphere (35 mm) is maintained above the surface of the drug solution (Figure 1).

(2) The transport cell is mounted on the electrode disk with or without a sheet of membrane (Figure 2) and then the whole transport cell-membrane-electrode combination is assembled on the rotating
Figure 8  The relation between the \( \log \left( \text{steady-state current} - \text{current at time } t \right) \) and time for p-Nitrophenol (5.56x10^-5M) diffusing through a Nuclepore membrane (41.6x10^-4 cm thick, 2.0 microns pore).
Figure 9  Relation between steady-state current and concentration of p-Nitrophenol at a rotating carbon disc electrode (3000 rpm) after successive addition of compound. The electrode is covered with a Nuclepore membrane (41.6x10^{-4} cm thick, 2.0 microns pore).
electrode body and rotated at a constant speed in the solution phase (Figure 1) for a given period of time to equilibrate the phases of solution, membrane and transport cell. Consideration is also given to the position of transport cell, the nitrogen pressure on the solution surface and the temperature of system.

(3) The polarogram is run under a varying applied potential or a constant applied potential. A current-potential curve (Figure 3) or a current-time curve (Figure 14) are recorded respectively on the chart of the automatic recorder or the screen of the oscilloscope system. The same steady-state current should be obtained at a given applied potential from these two profiles.

(4) The same procedure may be repeated for other solutions having a different drug concentration. A linear relationship should exist between the steady-state diffusion current ($i_{ss}$) and its corresponding drug concentration ($C_d$) as predicted by Eq. (13). A typical set of data is present in Figure 10. It is interesting to note that the linear relationship predicted by Eq. (13) is obeyed with and without a covering membrane of different thickness.
Figure 10  The linear relationship between diffusion current at steady-state ($i_{ss}$) and molar concentration of p-Nitrophenol in donor compartment ($C_d$) observed by single steady-state technique. The permeability may be estimated from the slope and is found to decrease as the thickness of covering Nuclepore membranes increases.
I. Model membranes for membrane permeation studies.

In general, two types of model membranes have been employed to study both non-steady-state and steady-state membrane permeation. To examine the effects of membrane thickness, Nuclepore membranes of the General Electric Co. were used. To investigate the importance of membrane pore area and tortuosity, Millipore membranes were used. In addition, Dialysis membranes of the Union Carbide Co. were also used in the protein binding studies which utilize the techniques developed in the membrane permeation studies.

The physico-chemical characteristics of these artificial membranes will be briefly discussed:

(1) Nuclepore membranes:

Nuclepore membranes are polycarbonate membranes produced by the General Electric Co..

The pores in these thin membranes, produced by exposing the membrane material to charged particles in a nuclear reactor and subsequent chemical etching, are uniform, smooth-bore, round holes which pass essentially straight through the membrane.

The most useful property of these membranes is its thinness, 7.6 to 11.7 x 10^{-4} cm. This and some of its other properties, e.g., transparent, tough, pliable and resistant to most common sol-
vents (except strong bases and halogenated hydrocarbons) make Nuclepore membranes useful in studying of the influence of membrane thickness on the mechanisms of membrane permeation.

(2) **Millipore membranes.**

Millipore membranes are porous structures composed of pure and biologically inert mixture of cellulose acetate and cellulose nitrate. These membranes are produced by Millipore Filter Co. and have the following useful physico-chemical properties: (i) The pores are uniform in size. They are currently available in 12 distinct pore sizes from 10 μm to 8 μm; (ii) High and constant porosity (80%) in all types; (iii) Constant thickness in all types (150 microns); (iv) Non-absorptivity; (v) Integral structure; (vi) High chemical resistance; and (vii) Thermal stability up to 125°C.

The uniformity in both porosity and thickness and the variety of pore sizes make Millipore membranes useful in the study of the effect of pore size on the mechanisms of membrane transport.

(3) **Dialysis membranes.**

Dialysis membrane is produced from cellulose by Union Carbide Co..

This membrane has a pore radius of 24 Å only and is permeable to water molecules and low mole-
cular weight compounds in aqueous solution while refusing passage to higher molecular weight substances, such as proteins and microorganisms. This makes it popular for use in protein-binding studies.

In an actual measurement of membrane permeation, the membrane sheet is presoaked in a refrigerated buffer system overnight before assembly on the rotating-disc electrode surface. Presoaking is necessary. Some examples are illustrated in Figure 11 where the time required to reach a steady-state level is a function of the pore size of Millipore membrane. The time needed to reach a steady-state is decreased with increased presoaking (Figure 12).

Even though, presoaking is not as important for both Nuclepore and Dialysis membranes, the overnight presoaking treatment was also applied to both of these membranes to provide constancy in experimental conditions.
Figure 11 The distribution of p-Nitrophenol ($4 \times 10^{-3}$M; pH 4.0; 30°C) in untreated Millipore membrane as function of the soaking time of membrane in drug solution (Rotation speed 600 rpm):
- SC (8 microns pore)
- HA (0.45 microns pore)
- VF (0.01 microns pore)
Figure 12  The distribution of p-Nitrophenol (2x10^{-3}M; pH 4.0; 30°C) in Millipore membrane type VF (1x10^{-6}cm pore) as function of presoaking the membrane in Acetate Buffer system:
- Untreated
- Acetate Buffer-treated, 2 hours
- Acetate Buffer-treated, overnight

ish: Spike height current
# RESULTS AND DISCUSSIONS

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A. Electrochemical reduction of Nitrophenols.

The electroreduction of the nitro group (-NO₂) in the Nitrophenol molecules is considered to go first to the Nitroso group (-NO), then to the hydroxylamine (-NHOH) and finally to the amine (-NH₂) (60-63, 66). This reaction scheme is shown in Figure 5 where two electrons are consumed for each molecule of Nitrophenol isomer in each step. Six electrons should be involved totally if the reduction goes to completion with the formation of amine.

In the studies on the polarographic behavior of mono-nitro aromatic compounds, Page and his associates (63) observed that two reduction waves were formed for both ortho- and para-Nitrophenols at pH 4.0 but for only para-Nitrophenol at pH 8.0. The first wave involves four electrons and indicates the reduction of nitro group up to hydroxylamine group only. The further reduction of the hydroxylamine group to the final product - amine which takes two more electrons gives the second wave. The half-wave potential, E½, of the second wave, however, is very negative and close to that at which potassium ion in the supporting electrolyte is reduced. Thus, the second wave is not clearly defined since a potassium-containing supporting electrolyte system is used in the present study.
As a rule, the reduction waves of organic substances, such as the Nitrophenol isomers studied in the present investigation (Figure 3), are governed by the rates of the electrode process and of the diffusion. For an irreversible wave as in the present case, the number of electrons determined from the slope of the linear log (i/\dot{i}d-i) - E plots is smaller than the actual number of electrons consumed in the electrode process (25).

As pointed out by Heyrovsky and Kuta (25), in any investigation of the mechanism of an electrode process, the number of electrons, \( n \), consumed by a single molecule or ion of the electroactive species on reduction or given up on oxidation must first be determined. A smaller number of electrons transferred in an irreversible process indicates that the rate of electrode process is slow. The slow electrode process results from the slow transition of the electroactive species to a form capable of electron exchange and requires a certain energy of activation. This slow electrode process is regarded as the cause of irreversibility.

However, the rate of the electrode process is a function of the potential applied to the system. It increases as the applied potential increases. Finally, a plateau is reached where the rate of electron-transfer is in equilibrium with the rate of diffusion, and the concentration of electroactive species at the electrode surface is essenti-
ally zero. At this moment, the steady-state diffusion current \( i_{ss} \) is diffusion-controlled in mechanism and defined by Nernst equation (Eq. 13).

\[
\begin{align*}
i_{ss} &= i_p - i_r \\
\text{and} \\
i_{ss} &= \frac{nFAD}{\delta} C_d
\end{align*}
\]

where \( i_p \) is the limiting current observed at the wave plateau and \( i_r \) the residual current from supporting electrolyte as defined earlier.

Now, the calculation of the actual number of electrons becomes possible. The number of electrons involved in the electroreduction of one Nitrophenol molecule in the present system, i.e., in K-acetate buffer (pH 4.0) or K-phosphate buffer (pH 7.4), is estimated, based on Eq. (13), from the steady-state diffusion current at the wave plateau and found to be four for para-, meta- and ortho-isomers with or without a covering membrane. This result is in harmony with the observations reported by Page et al. (63). It indicates that the Nitrophenol molecules are reduced at the electrode up to the intermediate N-hydroxylaminophenol (Figure 5).

It is apparent from the arguments discussed above that in order to approach a diffusion-controlled process where the rate of diffusion is the rate-determining step during the whole course of the electroreduction process and to
attain the real number of electrons involved in an electrode process, a potential in the plateau \((E_p)\) region should be used in the membrane permeation studies. When a constant plateau potential, e.g., -1.00 volt for Nitrophenols, is applied to the system, a time-independent steady-state current is observed soon after the polarograph circuit is switched on. The height of this steady-state current is defined by Equation (13). It is a diffusion-controlled current.

The electroreduction of aromatic nitro compounds is also very interesting with respect to the electron distribution in such compounds. The NO₂ group is a powerful electron-attracting group; consequently it is to be expected that the electron density in its vicinity will be affected by the nature and position of other electron-attracting or electron-donating substituents in the aromatic ring. This in turn will be reflected in the ease of electron capture at the electrode surface.

The hydroxyl group, which is an electron-donating group, at the para-position will increase the electron density of the nitro group. Thus, p-Nitrophenol is less readily reduced than m-Nitrophenol where the effect on the electron density of the NO₂ group is expected to be less. At a low pH where hydrogen bonding will nullify the increased electron density due to an ortho-nitro substituent, the o-Nitrophenol is less readily reduced than m-Nitrophenol.
These effects are clearly reflected in the electrochemical properties, i.e., decomposition potential ($E_d$) and halfwave potential ($E_{1/2}$), of these three isomers of Nitrophenol. Table 2 demonstrates that $E_d$ and $E_{1/2}$ of o- and m-Nitrophenol are very close and are significantly less negative than those for the p-isomer. Page et al. (63) also reported the same observation. Astle and McConnell (66) also rationalized that the intermolecular hydrogen bond formed between p-Nitrophenol and solvent molecules is the primary contributor to the more negative $E_{1/2}$ values observed in the p-Nitrophenol, and that the intramolecular hydrogen bonding that occurs in o-Nitrophenol results in the less negative $E_{1/2}$ value.
B. Linear diffusion-controlled processes

If the current produced from the electroreduction of Nitrophenols is controlled only, or primarily, by the rate of semi-infinite linear diffusion of Nitrophenol molecules, then according to Delahay's argument (27), this current is diffusion-controlled and described by the equation (20-2) developed previously

\[ i_Q = nFAD^\frac{1}{2} \Pi^{-\frac{1}{2}} C_d \ t^{-\frac{1}{2}} \]  

(20-2)

Plotting \( i_Q \) against \( t^{-\frac{1}{2}} \) should yield a straight line with a theoretical slope of \( nFAD^\frac{1}{2} \Pi^{-\frac{1}{2}} C_d \). Experimentally, it was observed that Eq. (20-2) is followed perfectly by all three isomers of Nitrophenol. A typical example is presented in Figure 13 for three concentrations of \( m \)-Nitrophenol. The values of the slope for a unit concentration are very constant and also close to the magnitude expected from theoretical considerations, e.g., for \( m \)-Nitrophenol, \( nFAD^\frac{1}{2} \Pi^{-\frac{1}{2}} \) is equal to \( 2.385 \times 10^3 \) \( \mu A \cdot \text{sec}^{-\frac{1}{2}} \cdot \text{M}^{-1} \). This result clearly demonstrates that the mass transport of Nitrophenol molecules from the bulk solution phase to the immediate surface of a stationary plane electrode disk is dominated by a linear-diffusion model. In addition, the intercept at the origin is also expected from Eq. (20-2).

Now, if this electrode disc is rotated, as in the present studies, at a synchronous speed, a forced convection
Figure 13 Relationship between the current with stationary electrode \( (i_Q) \) and the reciprocal of square root of time as described by Eq. (20). The resultant values of slope/conc. are: \( \bigcirc 1.98 \times 10^3 \), \( \bullet 2.10 \times 10^3 \), \( \circ 2.16 \times 10^3 \) \( \mu A \) sec. \(^{-1/2}\) M. Theoretical value should be \( 2.385 \times 10^3 \). The concentration of m-Nitrophenol is: \( \bigcirc 5 \times 10^{-3} \), \( \bullet 4 \times 10^{-3} \), and \( \circ 3 \times 10^{-3} \) M.
should result. Under this condition, the convective transport of Nitrophenol molecules to and from the electrode surface should be much faster than for natural convection, because the concentration gradient extends over a much thinner diffusion layer. According to the diffusion-layer model of Nernst (28) and Brunner (50, 51), this diffusion layer on the rotating-disc electrode surface should be static, that is within it the velocity of motion of the liquid is zero. If this is the case, then the rotation of the electrode functions simply to decrease the thickness of the diffusion layer and the $i - t^{1/2}$ linear relation should be observed. Experimentally, it is very important to check whether the mass transport of Nitrophenol molecules under this forced convection condition also follows the linear diffusion model as it does in the case of natural convection, to be certain that no other factors, e.g. surface diffusion or turbulence, are involved to complicate the mechanism of linear diffusion.

Experimentally, a different current-time curve will be seen for the electroreduction of Nitrophenols at a constant applied potential for a rotating-electrode than for a stationary electrode (Figure 14). For an electrode rotating at a synchronous speed, a steady-state flux is attained soon after the start of measurement; while for a quiet electrode, the current height decreases as a function of time. Both of these current heights can be expressed by the Nernst equa-
Figure 14 The current-time curves for the electroreduction of Nitrophenol isomers at an applied potential of -1.0 volt. A steady-state current flux may be easily obtained by rotating the electrode disc at a given rotation speed.
tion developed previously:

\[ i = nFAD \frac{C_d}{\delta} \]  \hspace{1cm} (13-1)

However, the thickness of diffusion layer on the surface of electrode, \( \delta \), is a time-independent parameter for the rotating electrode, and a time-dependent one for the stationary electrode as described before by Eqs. (21) and (19) respectively.

\[ \delta_R = 1.62 \frac{D^{\frac{1}{3}} v^{\frac{1}{6}}}{w^{\frac{1}{2}}} \]  \hspace{1cm} (21)

\[ \delta_Q = \Pi^{\frac{1}{6}} D^{\frac{1}{2}} t^{\frac{1}{2}} \]  \hspace{1cm} (19)

Substituting Eq. (21) into Eq. (13-1) gives

\[ i_R = (0.62 \ nFAD^{\frac{2}{3}} v^{\frac{1}{6}} C_d) \ w^{\frac{1}{2}} \]  \hspace{1cm} (22)

If the transport of Nitrophenol molecules under the forced convection conditions is also controlled by the rate of semi-infinite linear diffusion, then the current height, \( i_R \), should be a linear function of \( w^{\frac{1}{2}} \). Experimentally, this relationship is perfectly followed (Figure 18) and will be discussed further in Section (C) of this chapter.

Therefore, it is reasonable to conclude that the current height, \( i_R \), under forced convection also obeys the linear diffusion model without any complication or devia-
tion. The function of rotation is then simply to decrease the thickness of hydrodynamic diffusion layer and in this way to increase the current. The basic mechanisms of diffusion are not altered.

When an inert membrane with a porosity of £ covers the immediate surface of a rotating-disc electrode, the hydrodynamic diffusion layer is extended from the electrode surface to the membrane surface, i.e., $\delta_D$ is enlarged to $\delta_D + \delta_m$; and the diffusional area ($A$) available for drug transport will be decreased by a factor of $\varepsilon$ since only the membrane pore channels are open to the diffusion of drug molecules. Therefore, the current flux should be decreased by a factor of $\varepsilon$:

$$i_m = n F A \varepsilon D^1/2 \Pi^{-1/2} \sigma_d t^{-1/2}$$  \hspace{1cm} (25)

Experimentally, we observed that a linear relationship is obeyed between $i_m$, the current with a covering membrane, and $t^{-1/2}$ (Figure 15); the slope of which is equal to the slope without membrane (i.e., naked electrode) multiplied by the membrane porosity as expected from Eq. (25), e.g., 0.74 for Millipore membrane (type VC) and 0.2012 for Nuclepore membrane (1.0 micron pore). The intercept at origin is also expected from the theoretical model (Eq. 25).

Equation (25) may be extended to determine the porosity of a membrane with unknown porosity. For instance, the porosity of Dialysis membrane was determined to be 21.7%
Figure 15  Relation between current (i) and reciprocal of square-root of time for p-Nitrophenol (2x10^{-3} M, pH 4, 30°C). The observed values of slope/conc. \((x10^3 \mu a \text{ sec}^{-1} M^{-1})\) are:

- Without covering membrane, 2.89 (Theor. 2.87)
- With Nuclepore membrane, 0.603 (Theor. 0.577)
- With Millipore VC membrane, 2.23 (Theor. 2.13)
by this technique (Figure 16).

We have already discussed that the function of rotation is simply to decrease the thickness of hydrodynamic diffusion layer on the electrode surface. This hydrodynamic diffusion layer occurs on the membrane surface when a membrane sheet covers the immediate surface of the electrode.

All the experimental results reported in this section strongly demonstrate that the rotating-disc electrode is well behaved. The mass transport of Nitrophenol molecules from the bulk of the solution to the immediate surface of electrode disc under a synchronous rotation, either through a covering membrane or not, is essentially no different from a stationary electrode theoretically and experimentally. Both of them depend on the rate of semi-infinite linear diffusion. The function of rotation is simply to decrease the hydrodynamic diffusion layer thickness on the electrode surface and to increase the current flux. The membrane phase acts only as an physical barrier to the drug transport; so the relationship between the rate and amount of membrane permeation and the physical parameters, e.g., porosity, thickness, pore size and tortuosity, of membrane may be examined.
Figure 16 Linear diffusion of $K_2Fe(CN)_6$ (1x10$^{-2}$ M, pH 4, 30°C) through a Dialysis tubing (porosity is unknown). Theoretical slope/conc. value without a covering membrane is 0.96x10$^3$. The experimental porosity is calculated to be 21.7%.
C. Role of electrode rotation

The rotating-disc electrode, first developed and introduced by Levich (52) to obtain well-defined diffusion conditions, is a device which permits the use of a solid electrode on which a membrane can be positioned for measurements analogous to those of a dropping mercury electrode. It consists of a disc of metal which has one face exposed to the solution and is rotated around its central axis. Owing to the rotation, the solution is set into motion and flows past the boundary of the diffusion layer on the immediate surface of the electrode disc. Within this diffusion layer the velocity of motion of the solution is zero and the rate of diffusion of the electroactive species toward the electrode surface is also defined by the equation (13-1) as in the case of stationary electrode:

\[ i = nFAD \frac{C_d}{\delta} \]  

(13-1)

However, the thickness, \( \delta \), of diffusion layer here is no longer a time-dependent parameter as in the case of a stationary electrode but a time-independent parameter which is defined by

\[ \delta = 1.62 \frac{1}{D^\frac{1}{3}} \frac{1}{v^\frac{1}{6}} w^{-\frac{1}{2}} \]  

(21)
One obtains the following mathematical expression for the limiting current for a rotating-disc electrode by combining Eqs. (13-1) and (21)

\[ i_R = 0.62 \text{nFAD} \frac{2}{3} V^{-\frac{1}{6}} w^2 C_d \]  

(22)

Thus, a linear relationship should be followed between the diffusion current for a rotating-disc electrode, \( i_R \), and the bulk concentration of the electroactive species in the donor solution, \( C_d \), when all the other variables are constant. Figure 17 illustrates this relation for the various rotation speeds studied.

Equation (22) can be transformed to Eq. (23) to study the effect of the rotation speed on the current height per unit concentration of the electroactive species:

\[ \frac{i_R}{C_d} = (0.62 \text{nFAD} \frac{2}{3} V^{-\frac{1}{6}}) w^2 \]  

(23)

If the linearity of \( i_R/C_d \) to \( w^2 \) is perfectly obeyed, the slope of this plot should be equal to \( 0.62 \text{nFAD} \frac{2}{3} V^{-\frac{1}{6}} \). A typical example is shown in Figure 18. It is also noted that the experimental value of the slope is in good agreement with the value calculated from the theoretical slope, i.e., \( 8.98 \times 10^3 \, \mu A \, \text{sec}^{\frac{1}{6}} \, \text{M}^{-1} \).
Figure 17 The relation between steady-state diffusion current ($i_R$) and concentration of p-Nitrophenol at various rotation speeds (r.p.m.) of a carbon disc electrode.
The relation between steady-state current divided by concentration of p-Nitrophenol (concentrations 8, 20, and 40 x 10^{-4} M) and the square root of the angular rotation (revolution per sec.). The slope is 9.29 x 10^3 \mu A \sec^{-0.5}/M (Theoretical 8.98 x 10^3)
The advantages which the rotating-disc electrode offers are that the limiting current is time independent, very stable, and may be easily calculated from Eq. (22). Also, it is quite large compared with the dropping-mercury electrode, owing to the rapid rotation as illustrated in Table 1. Obviously, the diffusion current for a unit drug concentration is remarkably increased without sacrifice of electrochemical properties. The usefulness of a rotating-disc electrode becomes obvious.

Attention is also called to Eq. (21) where the thickness, $\delta$, of the diffusion layer on the electrode surface is inversely proportional to the reciprocal of the square root of angular rotation, $w^{-\frac{1}{2}}$. It is obvious that the increase in the magnitude of diffusion current, diagrammatically illustrated in Figure 18, with increasing rotation speed of the electrode is simply due to the decrease in the thickness of the diffusion layer as described by Eq. (13-1). For a given rotation speed, the magnitude of the diffusion layer thickness can be easily calculated from Eq. (21); for instance, for p-Nitrophenol in pH 4.0 Acetate Buffer system the thickness of diffusion layer is $11.0 \times 10^{-4}$ cm at a rotation speed of 3000 r.p.m. and is $16.1 \times 10^{-4}$ cm at 1200 r.p.m.
D. **Effects of membrane on the electrochemical behavior**

In addition to the advantages mentioned earlier that a rotating-disc electrode can offer, the smooth and flat electrode surface also allows a membrane sheet to be directly positioned on its immediate surface; thus, the solution volume of receiving compartment is negligibly small and the ratio of diffusional membrane area to compartmental volume is very large. Therefore, sensitivity of flux measurements in such a diffusional cell assembly will be improved, according to the arguments of Olson et al. (8) and of Flynn and Smith (7). Also, the assembling and cleaning of the transport cell becomes very convenient.

Manegold (32-35) has stated that a membrane phase acts simply to decrease the diffusional area available and to increase the effective length of diffusional path when it is covering the electrode surface. The assumptions made are that the membrane structure is porous and inert, that no physico-chemical interactions occur between penetrant species and membrane, and that membrane permeation is by way of the solution-filled pore channels. Experimentally, the decrease in the available diffusional area due to the covering of membrane was well related to membrane porosity in Section (B) (Figure 15). The increase in the effective length of diffusional path will be discussed in the following paragraphs.
In section (B), we have already discussed that a diffusion layer is present on the immediate surface of the electrode. The thickness of this diffusion layer ($\delta_D$) can be decreased by increasing the angular rotation speed ($w$) as described by Eq. (21):

$$\delta_D = 1.62 \frac{1}{D^3} \sqrt[3]{\frac{1}{6}} w^{-\frac{3}{2}}$$

(21)

When a membrane of thickness, $\delta_m$, is positioned on the electrode surface, the effective length of diffusion path for a molecule to diffuse will be extended from $\delta_D$ to $\delta_D + \delta_m$. So, the effective length of diffusion path can be expressed as

$$\delta = \delta_D + \delta_m$$

(44)

Therefore, both $\delta_D$ and $\delta_m$ contribute to the magnitude of $\delta$. Now, if the rotation speed of the electrode increases, the magnitude of $\delta$ will be proportionately decreased since the contribution from $\delta_D$ is decreased. The magnitude of $\delta_D$ may be decreased to a point after which the magnitude of $\delta_D$ is relatively small compared to $\delta_m$. The transport across the membrane then becomes the rate-limiting step in the whole course of transport across the diffusion path of thickness $\delta$.

Experimentally, we observed that this is the case. Figure 19 demonstrates this behavior. For a Dialysis membrane of pore radius $24 \times 10^{-8}$ cm and thickness $25.2 \times 10^{-4}$
Figure 19 The effect of the square root of angular rotation (revolution per second) on the steady-state current of p-Nitrophenol (1x10^{-4}M).

- Left ordinate without covering membrane.
- Right ordinate with a covering Dialysis membrane 25.2x10^{-4}cm (dry thickness), 24 Å pore radius.
cm, at a rotation speed of 600 r.p.m. or greater the current-rotation profile levels off. Any further increase in the rotation speed does not result in any significant increase in the current height since transport-across-membrane becomes the rate-limiting step after the rotation speed reaches a magnitude of 600 r.p.m. Thus, any study dealing with the mechanism of membrane transport should choose a rotation speed in this range in order to understand the real behavior of membrane transport.

Table 5 presents the data both of the steady-state permeability ($P_m$) and kinetics of membrane transport using two rotation speeds in the range where the transport-across-the membrane is the rate-determining step. Apparently, the 2.5-fold increase in the rotation speed, i.e., from 1200 to 3000 r.p.m. does not significantly change the magnitudes of both the steady-state permeability and the kinetics of membrane transport.

The membrane sheet is positioned on the electrode surface with the aid of a cell holder. Table 6 illustrates that the lightweight and inert holder does not influence the electrochemical properties of the diffusion-controlled electroreduction of Nitrophenols.
TABLE 5

THE EFFECT OF ROTATION SPEED OF THE ELECTRODE ON THE STEADY STATE PERMEABILITY AND THE KINETICS OF MEMBRANE TRANSPORT OF p-NITROPHENOL THROUGH DIALYSIS TUBING a

<table>
<thead>
<tr>
<th>Rotation Speeds (r.p.m.)</th>
<th>$\delta_D$ b (x10^-4 cm)</th>
<th>$(P_m)_E$ c (x10^-4 µA/M)</th>
<th>$k_m)_E$ d (sec.^-1)</th>
<th>$t_{1/2}$ (sec.)</th>
<th>$t_i$ (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000</td>
<td>11.0</td>
<td>4.33</td>
<td>0.2687</td>
<td>2.58</td>
<td>4.67</td>
</tr>
<tr>
<td>1200</td>
<td>16.1</td>
<td>4.26</td>
<td>0.2266</td>
<td>3.06</td>
<td>4.71</td>
</tr>
</tbody>
</table>

a Thickness of Dialysis tubing is 18.14 microns.
b Calculated from Eq. (21).
c 2.2 × 6.0 × 10^-4 M of p-Nitrophenol at pH 7.4
d 2.2 × 10^-4 M of p-Nitrophenol at pH 7.4
### TABLE 6

**THE ELECTROCHEMICAL PROPERTIES OF o-NITROPHENOL AT CARBON ELECTRODE SURFACE WITH TRANSPORT CELL IN POSITION**

<table>
<thead>
<tr>
<th>Electrochemical Properties*</th>
<th>Transport Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without</td>
</tr>
<tr>
<td>$i_{ss}$ (µamp.)</td>
<td>99.3</td>
</tr>
<tr>
<td>$E_d$ (volts)</td>
<td>-0.47</td>
</tr>
<tr>
<td>$E_{1/2}$ (volts)</td>
<td>-0.645</td>
</tr>
</tbody>
</table>

* 1 x $10^{-4}$ M of o-Nitrophenol in pH 4.0 Acetate buffer, Beckman carbon electrode at 3000 rpm and 30°C.
E. Membrane permeation studied by "Single Steady-state Technique"

The "Single Steady-state Technique" at a constant applied potential, which was described in the chapter of "Methodology" under the section of "Technology of Measurement", is utilized here to study the permeation of three types of model membranes to aromatic nitro compounds, e.g., Nitrophenols, Nitroanilines and Nitrobenzoic acids.

One of the purposes for performing these preliminary investigations is to have a better understanding of the applicability of this new "Rotating-disc Electrode Polarograph System" to the studies of membrane permeation to drug molecules.

(1) Permeation of Dialysis membrane to Aromatic Nitro Compounds.

In order to make a comparison of the membrane permeability between drug molecules with different stereochemistries, a parameter called permeability constant, which is the ratio of the diffusion current (or permeability) with membrane to that without membrane, is used.

In these studies, a Dialysis membrane (48 x 10^-8 cm pore) is used since its pore diameter is only 6-fold the molecular size of Nitrophenol molecules. Therefore, any effect of membrane struc-
ture or any interaction between drug molecule and membrane phase may be easier to observe. In addition, a series of three aromatic nitro compounds was investigated in order to compare the effect of the electron-seeking nitro and carboxyl groups with that of the electron-donating hydroxyl and amino groups. Results are listed in Table 7.

A comparison made between the data in the last two columns and those in the third column demonstrates that the permeation of all aromatic nitro compounds is diffusion-controlled, even though the pore diameter of Dialysis membrane is only 6-fold the molecular size of the aromatic nitro compounds and the stereochemical characteristics of these model drugs are quite different from one another. The larger the diffusivity of a penetrant species, the higher is its membrane permeability. The diffusion pattern is also followed using two thicknesses of Dialysis membrane, even though membrane permeability is decreased by increasing the membrane thickness.

All the aromatic nitro compounds investigated were dissolved in a pH 4.0 Acetate Buffer system. Under this condition, all the molecules are present as unionized species in the solution. Although the difference in the relative electronegativity
### TABLE 7

**THE EFFECT OF DIFFUSION COEFFICIENT ON THE PERMEABILITY OF AROMATIC NITRO COMPOUNDS THROUGH DIALYSIS MEMBRANE (pore size 48 Å)**

<table>
<thead>
<tr>
<th>Model Drugs</th>
<th>$10^{-6}D^a$ (cm²/sec.)</th>
<th>Ratio $b$</th>
<th>Permeability Constant $c$</th>
<th>Ratio $b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrophenol</td>
<td></td>
<td></td>
<td>A $d$</td>
<td>B $d$</td>
</tr>
<tr>
<td>p-</td>
<td>9.18</td>
<td>1.00</td>
<td>0.056 0.046</td>
<td>1.00 1.00</td>
</tr>
<tr>
<td>o-</td>
<td>7.76</td>
<td>0.85</td>
<td>0.046 0.039</td>
<td>0.82 0.85</td>
</tr>
<tr>
<td>m-</td>
<td>6.34</td>
<td>0.69</td>
<td>0.041 0.033</td>
<td>0.73 0.72</td>
</tr>
<tr>
<td>Nitroaniline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-</td>
<td>9.91</td>
<td>1.05</td>
<td>0.052 0.045</td>
<td>0.93 0.98</td>
</tr>
<tr>
<td>m-</td>
<td>7.44</td>
<td>0.81</td>
<td>0.044 0.038</td>
<td>0.79 0.83</td>
</tr>
<tr>
<td>Nitrobenzoic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-</td>
<td>6.22</td>
<td>0.68</td>
<td>0.037 0.030</td>
<td>0.66 0.65</td>
</tr>
<tr>
<td>o-</td>
<td>6.34</td>
<td>0.69</td>
<td>0.031 0.025</td>
<td>0.55 0.54</td>
</tr>
</tbody>
</table>

---

*a* From literature: References (63) and (98).

*b* Using p-Nitrophenol as the reference.

*c* Diffusion current with membrane divided by diffusion current without membrane.

*d* Dialysis membrane. Thickness for types A and B are $27.7 \times 10^{-4}$ and $40.3 \times 10^{-4}$ cm respectively.
and in the stereochemical position of functional groups will result a different $\pi$-electron distribution or delocalization in the aromatic ring, no electrostatic interaction from the so-called "surface negative charge" on the Dialysis membrane can be detected in the present studies. Therefore, in the following membrane permeation studies, the solution of model drugs is controlled (buffered) at pH 4.0 to prevent possible complications from electrostatic interactions.

(2) Permeation of Nuclepore membranes to Nitrophenols

The relatively thin Nuclepore membranes are an ideal barrier to study the effect of membrane thickness on the permeation of drugs. Several sheets of membrane may be assembled together to lengthen the effective thickness for diffusion. Figure 10 presents a typical set of data where the linear relationship is obeyed for all the permeation studies with and without membranes. From the slope of these straight lines, steady-state permeability data can be estimated. It is obvious that the steady-state permeability is decreased considerably due to the presence of membrane phase. The steady-state membrane permeability is even decreased more as the number of Nuclepore membranes assembled is increased. In other words, the steady
-state membrane permeability is inversely proportional to the membrane thickness. This relation is followed for all three isomers of Nitrophenol compound as demonstrated in Table 8.

Furthermore, the permeability constant, which is the ratio of the steady-state permeability with membrane ($P_m$) to that without membrane ($P_o$), is also decreased as the membrane thickness increases.

We have already derived the following equations to describe the relationship between steady-state permeability and thickness for the cases where a membrane is or is not covering the electrode surface:

$$\log P_o = \log (nFA_oD_o) - \log \delta_D$$  \hspace{1cm} (26-1)

$$\log P_m = \log (nFA_mD_m) - \log (\delta_D + \delta_m)$$  \hspace{1cm} (26-2)

where the subscripts o and m denote that without and with a covering membrane. The permeability constant ($P_m/P_o$) can be derived from Eqs. (26-1) and (26-2)

$$\log \frac{P_m}{P_o} = \log \frac{A_mD_m}{A_oD_o} - \log \frac{\delta_D + \delta_m}{\delta_D}$$  \hspace{1cm} (45)

Based on Eq. (45), it is apparent that a straight line with theoretical slope of -1 should result when $\log (P_m/P_o)$ is plotted against $\log
### TABLE 8

THE EFFECT OF MEMBRANE THICKNESS ON THE STEADY-STATE PERMEABILITY OF NITROPHENOLS

<table>
<thead>
<tr>
<th>Nitrophenols</th>
<th>Membrane Thickness (x10⁻⁴ cm)</th>
<th>Permeability (x10⁻² μa/M)</th>
<th>Permeability Constant</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>para -</td>
<td>0.0</td>
<td>8.0</td>
<td>1.0</td>
<td>- 0.99</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>1.407</td>
<td>0.176</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.8</td>
<td>0.933</td>
<td>0.117</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34.2</td>
<td>0.73</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td>ortho-</td>
<td>0.0</td>
<td>7.0</td>
<td>1.0</td>
<td>- 1.09</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>1.2</td>
<td>0.171</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.8</td>
<td>0.77</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34.2</td>
<td>0.60</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
<td>meta -</td>
<td>0.0</td>
<td>7.4</td>
<td>1.0</td>
<td>- 1.17</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>1.28</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.8</td>
<td>0.65</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34.2</td>
<td>0.47</td>
<td>0.063</td>
<td></td>
</tr>
</tbody>
</table>

a 1.0~10.0 x 10⁻⁴ M of Nitrophenol at pH 4.0 and 30°C.

b The ratio of permeability with membrane to that without membrane.

c Estimated from Eq. (45). Theoretical slope should be - 1.0
Experimentally, this linear relation is followed perfectly for all three Nitrophenol isomers as shown in the last column of Table 8.

Both para- and ortho-isomers of Nitrophenol give a slope almost equal to the theoretical value of -1. But m-Nitrophenol yields a steeper slope which indicates that this isomer may show a small degree of a barrier effect due to its stereochemical structure. This finding agrees perfectly with the results obtained from "Consecutive Steady-state Technique" reported later.

Table 9 shows another set of typical data of steady-state membrane permeability. The effect of membrane thickness on the permeation of p-Nitrophenol at steady-state is in a same direction for all three Nuclepore membranes with different pore size and porosity. The linear relationship of the steady-state membrane permeability ($P_m$) to the reciprocal of the thickness of diffusion path, \( \frac{1}{\delta_D + \delta_m} \), as predicted from Eq. (18-2) is followed for all the Nuclepore membranes studied.

\[
P_m = \frac{n\text{FAD}}{(\delta_D + \delta_m)} 
\]  

(18-2)
### TABLE 9

**THE EFFECT OF MEMBRANE POROSITY (ε) ON THE PERMEATION OF p-NITROPHENOL THROUGH NUCLEPORE**

<table>
<thead>
<tr>
<th>Membrane&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ε&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>δ&lt;sub&gt;m&lt;/sub&gt; (x10&lt;sup&gt;4&lt;/sup&gt; cm)</th>
<th>P&lt;sub&gt;m&lt;/sub&gt; (x10&lt;sup&gt;-3&lt;/sup&gt; μa/cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>nFAD</th>
<th>ε&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Theoret. Experi.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENP 08</td>
<td>20.12</td>
<td>11.4</td>
<td>1.24</td>
<td>5.0</td>
<td>1.01</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.8</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.2</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GENP 05</td>
<td>23.56</td>
<td>11.7</td>
<td>1.51</td>
<td>4.1</td>
<td>0.97</td>
<td>23.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.4</td>
<td>0.92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35.1</td>
<td>0.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GENP 10</td>
<td>31.4</td>
<td>10.2</td>
<td>1.9</td>
<td>3.0</td>
<td>0.94</td>
<td>31.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.4</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.6</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Nuclepore membranes of pore sizes: 5x10<sup>-5</sup>, 8x10<sup>-5</sup> and 10x10<sup>-5</sup> cm.

<sup>b</sup> From manufacturer's specifications.

<sup>c</sup> 1.0 6.0 x 10<sup>-3</sup> M of p-Nitrophenol at pH 4.0 and 30°C.

<sup>d</sup> Estimated by dividing the experimental nFAD over theoretical values.
The slope of these linear plots, nFAD, may be a valuable indicator on the effect of membrane if there is any. The values of experimental slope listed in the 6th column of Table 9 indicate that the magnitude of nFAD decreases as the membrane porosity, calculated from manufacturer's data, increases. From the ratio of experimental nFAD over theoretical data, the experimental values of membrane porosity may be estimated. The results are listed in the last column of Table 9. It is noted that the experimental porosities coincide with the manufacturer's specifications perfectly. This result clearly indicates the importance of membrane porosity in the determination of steady-state membrane permeability, that is, the larger the membrane porosity, the higher the steady-state membrane permeability.

Apparently, these nFAD values should be multiplied by the respective membrane porosity (ε) to account for the decrease in the area (A) available for the diffusion of drug molecules.

The thickness of membrane in the present investigation is increased by overlapping one membrane on others of the same type. The thickness can be increased two to ten-fold simply by increasing the number of membranes. The obedience of
theoretical slope ($n_{FAED}$) in these multiple membranes indicates that permeation through these porous membranes is a diffusion controlled process (99). The kinetics of membrane transport, which will be discussed later, also support this point of view.

(3) Permeation of Millipore membranes to Nitrophenols

The fact that Millipore membranes have the same porosity (80%) and a constant thickness (150 microns), but a wide range of pore sizes (from $1 \times 10^{-6}$ to $8 \times 10^{-4}$ cm) makes them the ideal barrier in the investigation of the influence of the membrane pore size on the permeation of drug molecule.

A typical set of permeation data are tabulated in Table 10 which demonstrate that the smaller the membrane pore size, the lower the membrane permeability at steady-state. The present observation coincides with that reported by Uzelac and Cussler (30) and is also anticipated on the ground of Manegold's conclusion (32-35).

The significance of membrane thickness to the membrane permeability at steady-state is also examined in these thick membranes. A representative set of data are shown in Figure 20. Apparently, the relationship between the steady-state membrane permeability and the thickness is followed also in
TABLE 10

EFFECT OF PORE SIZE IN MILLIPORE MEMBRANES ON THE STEADY-STATE PERMEABILITY OF p-NITROPHENOL AT pH 4.0 STUDIED BY "SINGLE STEADY-STATE TECHNIQUE"

<table>
<thead>
<tr>
<th>Millipores</th>
<th>Pore Size (x10^-4 cm)</th>
<th>Steady-state Permeability (x10^-3 mA/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5400.0</td>
<td>9.0</td>
</tr>
<tr>
<td>SC</td>
<td>8.0</td>
<td>2.2</td>
</tr>
<tr>
<td>HA</td>
<td>0.45</td>
<td>1.3</td>
</tr>
<tr>
<td>VC</td>
<td>0.10</td>
<td>0.78</td>
</tr>
<tr>
<td>VF</td>
<td>0.010</td>
<td>0.68</td>
</tr>
</tbody>
</table>
Figure 20 The linear relationship between diffusion current at steady-state ($i_{ss}$) and concentration of p-N02-phenol in donor compartment ($C_d$) observed with Millipore membrane type HA. When membrane thickness increases from $150 \times 10^{-4}$, $300 \times 10^{-4}$, to $450 \times 10^{-4}$ cm, the steady-state membrane permeability decreases from $4.74 \times 10^3$, $3.41 \times 10^3$, to $2.97 \times 10^3$ $\mu$A/M. No covering membrane is also included for comparison. Its permeability is $27.5 \times 10^3$ $\mu$A/M.
these thick membranes as in the case of thin Nuclepore membranes discussed earlier, that is the membrane permeability at steady-state is inversely proportional to the membrane thickness; the thicker the membrane, the lower the membrane permeability.
F. Membrane permeation studied by "Consecutive Steady-state Technique"

The application of "Single Steady-state Technique" to the studies of membrane permeation has provided strong evidence testifying to the usefulness and applicability of the "Rotating-disc Electrode Polarograph System" in membrane transport measurements. The most significant advantages proved to be: (1) The membrane phase can be directly positioned on the smooth electrode surface without sacrificing its physical texture, therefore cell sensitivity is remarkably high and the time required to reach the steady-state flux is short; (2) Flux in membrane transport can be measured directly, so conventional sampling procedures no longer become necessary; (3) Reproducibility is quite good, since a constant diffusion-layer thickness is maintained by rotating the electrode-membrane-transport cell combination at a high synchronous speed; and (4) The assembly of membrane and the washing of transport cell become convenient. The technique of single steady-state is really very useful in studying the permeation of drug species at steady-state through membranes.

However, an advanced methodology called "Consecutive Steady-state Technique" was developed. The use of this new technique advanced the versatility of the "Rotating-disc Electrode Polarograph System". Now, both the steady-state
membrane permeability and the dynamics of membrane transport can be measured simultaneously and continuously in a single measurement. In addition, the concomitant use of a high chart-speed automatic recorder enables this methodology to provide insights into transient phenomena which might be significant to the mechanism of membrane permeation but which could be overlooked using the conventional technique involving a slow and interrupted sampling procedures.

The basis on which the "Consecutive Steady-state Technique" is designed is the following: the membrane and its holder in supporting electrolyte is first allowed to come to equilibrium, then it is disturbed by suddenly increasing the drug concentration in the donor solution compartment, so that it is no longer at equilibrium. The rate with which the system approaches its new equilibrium is then followed using sensitive polarographic instrumentation. The procedure used has already been summarized in the chapter of "Methodology" under the section of "Technology of measurement". Its instrumentation has also been discussed elsewhere in the same chapter. Figure 7 is a representative observation typifying that obtained on the recorder.

The current-time profile as in Figure 7 will provide us with two sets of useful information about the mechanism of membrane permeation: the dynamics of membrane transport
at the non-steady-state and the membrane permeability at the steady-state.

The dynamics of membrane transport at non-steady-state may be understood by plotting $\log (i_{ss} - i_t)$ against time based on Eq. (17) developed previously.

$$\log (i_{ss} - i_t) = \text{constant} - \frac{km}{2.303} t \quad (17)$$

where $km$ was defined before by Eq. (16-2)

$$km = \frac{\Pi^2 D}{(\delta_D + \delta_m)^2} \quad (16-2)$$

A typical plot is shown in Figure 8. From this first-order type plot, the rate constant for membrane transport ($km$) may be obtained from the slope of the linear portion. The half-life, $t_{\frac{1}{2}}$, to reach the steady-state is defined in the usual way:

$$t_{\frac{1}{2}} = \frac{0.693}{km} \quad (46)$$

Secondly, all the observed steady-state values for current in Figure 7 after correction for a common residual current (Eq. 40) should be directly proportional to the corresponding drug concentration in the donor compartment after each increase in concentration. According to the theoretical model developed earlier, Eq. (13) should be obeyed.
A linear relationship exists between the steady-state current ($i_{ss}$) and the drug concentration in the donor solution ($C_d$). Figure 9 demonstrates that the experimental data follow this linearity. The slope of this linear plot is here defined as the steady-state permeability ($P_m$)

$$\frac{i_{ss}}{C_d} = P_m = \frac{nFAD}{(\delta_D + \delta_m)}$$  

The theoretical slope, $nFAD/(\delta_D + \delta_m)$, for a naked electrode with a rotation speed at 3000 r.p.m. is estimated to be $7.38 \times 10^{-5}$ mA/m. The experimental slope calculated in Figure 6 is found in good agreement with this theoretical calculation.

Furthermore, Eq. (16-2) indicates that the rate-constant for membrane transport ($k_m$) is inversely proportional to the square of effective length of diffusion path, $(\delta_D + \delta_m)^2$; and Eq. (18-2) tells us that the steady-state permeability ($P_m$) is a linear function of the reciprocal of $(\delta_D + \delta_m)$. Figures 21 and 22 show that both $k_m$ and $P_m$ decrease with an increase in membrane thickness ($\delta_m$) which in turn make the effective length of diffusion path, $(\delta_D + \delta_m)$, larger. The effect of membrane thickness on the steady-
Figure 21  Linear relationship between Log (i_{ss} - i_t) and time as described by Eq. (17). The slope of these straight lines decreases with increasing thickness of Nuclepore membranes: □ $11.7 \times 10^{-4}$ cm, ○ $23.4 \times 10^{-4}$ cm and ○ $35.1 \times 10^{-4}$ cm.
Figure 22 Linear relationship between $i_{ss}$ and $C_d$ as described by Eq. (13). The slope of these straight lines decreases as the thickness of Nuclepore membranes increases: $11.7 \times 10^{-4}$, $23.4 \times 10^{-4}$, $35.1 \times 10^{-4}$, $46.8 \times 10^{-4}$, and $58.5 \times 10^{-4}$ cm.
state membrane permeability and the kinetics of membrane transport is also illustrated in Table 11 for five membrane thicknesses.

Before getting into a detailed discussion on the effect of membrane thickness, it is important to see whether Eq. (45), which has been shown to be valid in the "Single Steady-state Technique" (Table 8), is also obeyed when the "Consecutive Steady-state Technique" is employed. The experimental data on the steady-state membrane permeability listed in Table 11 are also utilized to calculate the permeability constants and plotted as before in Figure 23 based on Eq. (45). The resultant slopes of both para- and ortho-Nitrophenols are close to the theoretical slope of -1 as expected from Eq. (45), but meta-isomer shows a slight deviation, i.e., -1.12.

The results in Figure 23 perfectly coincide with the observation reported in Table 8. This agreement strongly demonstrates the validity of the theoretical model for membrane permeation developed in the present studies and also clearly indicates the applicability of this new and advanced technique of "Consecutive Steady-state" in the membrane transport studies.
### TABLE 11

**EFFECT OF MEMBRANE THICKNESS ON THE DYNAMICS OF MASS TRANSPORT OF p-NITROPHENOL THROUGH THE NUCLEOFOR Membranes**

<table>
<thead>
<tr>
<th>Thickness (x10^-4cm)</th>
<th>Pm (x10^-4µa/M)</th>
<th>km (second^-1)</th>
<th>t½ (seconds)</th>
<th>t½ (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>78.2</td>
<td>Too fast to be recorded</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>11.7</td>
<td>8.02</td>
<td>1.0895</td>
<td>0.64</td>
<td>1.27</td>
</tr>
<tr>
<td>23.4</td>
<td>5.37</td>
<td>0.6319</td>
<td>1.10</td>
<td>2.06</td>
</tr>
<tr>
<td>35.1</td>
<td>4.00</td>
<td>0.2950</td>
<td>2.35</td>
<td>2.48</td>
</tr>
<tr>
<td>46.8</td>
<td>3.63</td>
<td>0.2374</td>
<td>2.92</td>
<td>4.13</td>
</tr>
<tr>
<td>58.5</td>
<td>2.77</td>
<td>0.1536</td>
<td>4.51</td>
<td>4.64</td>
</tr>
</tbody>
</table>

---

**a** Pore size: 0.5 microns.

**b** 5.56~18.9 x 10^-5 M of p-Nitrophenol at pH 4.0 and 30°C.

**c** 5.56 x 10^-5 M of p-Nitrophenol at pH 4.0 and 30°C.

**d** Induction time which is the time elapsed before the drug molecules first appear in the receiving compartment.
Permeability Constant

\[
\frac{(\delta_D + \delta_m)}{\delta_D}
\]

Figure 23 Relationship between log (permeability constant) and log (thickness ratio) for all Nitrophenol isomers. The resultant slopes are: ◆ para, -1.01, • ortho, -1.03; ○ meta, -1.12. Theoretical value should be -1.00. Concentration of Nitrophenol is 5.56 \(\pm\) 15.0 \(\times\) 10\(^{-5}\) M, pH 4.0, 30°C.
G. Nuclepore membrane and membrane permeation

Theoretically, the steady-state membrane permeability, $P_m$, is inversely proportional to the sum of diffusion layer thickness and membrane thickness, $(\delta_D + \delta_m)$, as described earlier in Eq. (18-2)

$$P_m = \frac{nFAD}{\delta_D + \delta_m}$$

(18-2)

Since the thickness of hydrodynamic diffusion layer, $\delta_D$, is a constant value fixed by the rotating-disc electrode, the only variable in Eq. (18-2) is $\delta_m$. Therefore, increasing the thickness of membrane, $\delta_m$, will decrease the magnitude of $P_m$. The linear relationship is shown in Figure 24. The slope of this straight line should be equal to $nFAD$ as described in Eq. (18-2). In the present system, the same number of electrons are involved in the reduction of the Nitrophenol isomers and the same membrane area is available for permeation; thus, the diffusivity, $D$, of the Nitrophenols is the sole variable determining the difference in the magnitude of the theoretical slope. The diffusivities calculated from Page et al. (63) are employed to compute the theoretical slopes based on $nFAD$. The results have been tabulated in Table 12 together with the experimental slope data.
Figure 24 Effect of membrane thickness on the steady-state membrane permeability ($P_m$) as described by Eq. (18-2). The observed slope (nFAD) is $197.1 \, \mu\text{A-cm/M}$ for p-Nitrophenol ($5.56 \sim 15.0 \times 10^{-5}$M; pH 4.0; 30°C) and Nucleopore membrane ($5 \times 10^{-5}$ cm pore; $11.7 \sim 58.5 \times 10^{-4}$ cm); $\delta_D$ is $11.0 \times 10^{-4}$ cm at a rotation speed of 3000 rpm.
TABLE 12

THE EFFECT OF MEMBRANE POROSITY ON THE PERMEATION OF NITROPHENOL THROUGH NUCLEPORE MEMBRANES

<table>
<thead>
<tr>
<th>Nitrophenols</th>
<th>$\frac{10^6 D}{(\text{cm}^2/\text{sec.})}$</th>
<th>nFAD ((\mu\text{a-cm/M}))</th>
<th>(\epsilon) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-</td>
<td>9.18</td>
<td>822.21</td>
<td>197.1</td>
</tr>
<tr>
<td>o-</td>
<td>7.76</td>
<td>695.03</td>
<td>213.3</td>
</tr>
<tr>
<td>m-</td>
<td>6.34</td>
<td>567.84</td>
<td>173.3</td>
</tr>
</tbody>
</table>

\(\text{a}^{5.56 - 15.0 \times 10^{-5}}\) M of Nitrophenol at pH 4.0 and 30°C.

\(\text{b}^{\text{Calculated from Reference (63).}}\)

\(\text{c}^{\text{Estimated by dividing the experimental nFAD over theoretical values.}}\)

\(\text{d}^{\text{nFAD = } 4 \times 9.6514 \times 10^7 \times 0.232 \times D = 89.565 \times D (\mu\text{a-cm/M})}\)
It is apparent that the experimental slopes are quite small compared with those calculated from the theoretical model. A possible reason for such a deviation is that when an electrode is covered with a sheet of membrane, the area (A) available for the diffusion of Nitrophenol molecules is decreased by the porosity (ε) of the membrane since only the water-filled pore channels are available for membrane permeation. This was already recognized in Table 9. In the present system, the importance of membrane porosity in the steady-state membrane permeability may be appreciated by comparing experimental slopes with corresponding theoretical slopes. The ratio of these slopes are listed in the last column of Table 12. It is interesting to note that these experimental porosity data are very close to the porosity, i.e., 24%, estimated from the manufacturer's specifications of pore size and pore density. The porosity estimates made from the manufacturer's specifications are crude estimates at best.

Therefore, when a membrane exists, the membrane porosity (ε) should be included in Eq. (18-2) to account for the decrease in the available diffusion area, thus

\[ P_m = \frac{nFAD\varepsilon}{(\delta_D + \delta_m)} \]  

(47)
As mentioned before, the rate-constant for membrane transport \((k_m)\) is theoretically inversely proportional to the square of the sum of diffusion layer thickness and membrane phase thickness, \((\delta_d + \delta_m)^2\), as described by

\[
k_m = \frac{\Pi^2D}{(\delta_d + \delta_m)^2}
\]  

(16-2)

The same argument discussed earlier is also applicable in the present analysis. A linear relationship is predicted when \(k_m\) is plotted against \((\delta_d + \delta_m)^{-2}\), and the slope of the resultant straight line, \(\Pi^2D\), will be a function of the diffusion coefficient \((D)\) of the penetrant molecules. Figure 25 is a typical plot illustrating this relation.

The same set of diffusion coefficient data used in calculating \(nFAD\) (Table 12) is also utilized here to compute the theoretical slope of \(\Pi^2D\). The resultant values of \(\Pi^2D\) are tabulated together with the experimental slopes in Table 13.

The data in the last column of Table 13 are the ratio of the theoretical slopes to their corresponding experimental values. These data clearly indicate that all the experimental slopes with the Nitrophenol isomers are much lower than those values expected from the theoretical model. In the case of ortho-isomer, the deviation is even greater compared to those for para- and meta-Nitrophenols. It was repor-
Figure 25  Effect of membrane thickness on the rate-constant for membrane transport ($k_m$) as described by Eq. (16-2). The observed slope ($\pi^2 D$) is $1.9541 \times 10^{-5}$ cm$^2$/sec. for o-Nitrophenol ($5.56 \times 10^{-5}$ M) and Nuclepore membrane (pore size: $5 \times 10^{-5}$ cm; thickness: $11.7 \sim 58.5 \times 10^{-4}$ cm). $\delta_D = 10.83 \times 10^{-4}$ cm at a rotation speed of 3000 rpm.
TABLE 13

A COMPARISON OF THE EXPERIMENTAL SLOPE OF PLOTS OF $k_m$ vs. 
$(1/(\delta_p + \delta_m)^2)$ WITH THE THEORETICAL SLOPE ($\pi^2 D$)

<table>
<thead>
<tr>
<th>Nitrophenols</th>
<th>$10^6 D$</th>
<th>$\pi^2 D$ (x10$^6$ cm$^2$/sec.)</th>
<th>Ratio$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cm$^2$/sec.)</td>
<td>Theoretical</td>
<td>Experimental</td>
</tr>
<tr>
<td>para -</td>
<td>9.18</td>
<td>90.60</td>
<td>5.38</td>
</tr>
<tr>
<td>meta -</td>
<td>6.34</td>
<td>62.57</td>
<td>3.73</td>
</tr>
<tr>
<td>ortho-</td>
<td>7.76</td>
<td>76.59</td>
<td>1.95</td>
</tr>
</tbody>
</table>

$^a$ $5.56 \times 10^{-5}$ M of Nitrophenol at pH 4.0 and 30°C.

$^b$ Calculated from Reference (63).

$^c$ Theoretical slope = $\pi^2 D = 9.87 \times D$.

$^d$ Estimated by dividing the theoretical $\pi^2 D$ over experimental values.
ted earlier (Tables 8, 9 & 12, Figure 23) that at steady-state, the membrane permeability of all three Nitrophenol isomers showed obedience to theory even though a literature value of diffusivity was used in the calculation. Therefore, it seems that the mechanism of membrane permeation at non-steady-state is quite different from that at steady-state.

Table 14 presents data to show the difference in mechanisms of membrane permeation at non-steady-state from that expected. The agreement of experimental steady-state membrane permeability with theoretical values is immediately recognized. On the other hand, the degree of deviation of \((k_m)_E\) from \((k_m)_T\) appears to be a function of the pore size and/or the porosity of Nuclepore membrane, that is, the smaller the pore size and/or the porosity, the larger the deviation. The dependency of the kinetics of membrane transport on porosity could rise from a number of phenomena: the "Restricted flow" model of Lonsdale et al. (99) and the concentration gradient-dependency of diffusivity theory (100) are two of those possible explanations. Lonsdale et al. observed that the rate of water flow through a membrane was lower than that expected from theoretical calculations. The deviation in water flux from theory was found to be a function of porosity in the membrane; when the porosity is lower than 23 %, the flow rate is totally restricted and when higher than 23 %, the flow rate is partially restrict-
TABLE 14

THE DEGREE OF DEVIATION OF THE DYNAMICS OF MEMBRANE TRANSPORT OF NITROPHENOLS\textsuperscript{a} THROUGH NUCLEPORE MEMBRANES AS A FUNCTION OF PORE SIZE AND POROSITY

<table>
<thead>
<tr>
<th>Pore Size (x10^{-4} cm)</th>
<th>$\varepsilon^b$ (%)</th>
<th>$(F_m)_E/(F_m)_T$ para-</th>
<th>$(F_m)_E/(F_m)_T$ ortho-</th>
<th>$(k_m)_E/(k_m)_T^c$ para-</th>
<th>$(k_m)_E/(k_m)_T^c$ ortho-</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>9.43</td>
<td>1.03</td>
<td>0.95</td>
<td>0.042</td>
<td>0.031</td>
</tr>
<tr>
<td>0.50</td>
<td>23.6</td>
<td>1.22</td>
<td>0.94</td>
<td>0.141</td>
<td>0.117</td>
</tr>
<tr>
<td>1.00</td>
<td>31.4</td>
<td>0.90</td>
<td>1.05</td>
<td>0.193</td>
<td>0.164</td>
</tr>
<tr>
<td>2.00</td>
<td>50.3</td>
<td>1.14</td>
<td>1.14</td>
<td>0.291</td>
<td>0.257</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 5.56 \sim 15.0 \times 10^{-5} \text{ M of Nitrophenol at pH 4.0, 30°C and 3000 r.p.m.}.

\textsuperscript{b} Calculated from Manufacturer's specifications.

\textsuperscript{c} Estimated from Eq. (16-2)
ed until unlimited flux is reached with 100% porosity. Because of the fact that all the Nuclepore membranes in the present study contain a porosity lower than 50%, a restriction on the membrane transport of Nitrophenol molecules may be expected (Table 14). The decrease in rate due to restricted flow, which essentially results from an increased diffusional path length, may explain the rate data, however, we can not explain the agreement of experimental $P_m$ with theoretical values (Tables 12 & 14) (Since $P_m$ is also length-dependent). If we involve the concentration gradient-dependency of diffusivity theory (100), that is, the diffusivity is a function of the concentration gradient across the membrane phase; then, at non-steady-state, the concentration gradient may be time-dependent while at steady-state, a constant concentration gradient is attained. Thus, we may not expect the same diffusivity to operate at both non-steady-state and steady-state membrane permeation. Furthermore, the pore size and/or porosity of membrane can be important parameters in attaining a constant concentration gradient, and thus, in turn, affect the rate at which steady-state diffusion is obtained.

However, these two arguments could not be extended to the observations made in Millipore membranes in which both $P_m$ and $k_m$ values were found to decrease as the membrane pore size decreases (Table 16). The decrease in both $P_m$ and $k_m$ data has been related through the tortuosity (Tables 17
& 18) of the membrane. Using the tortuosity calculated from steady-state membrane permeability, the experimental values in the rate-constant for membrane transport correlate perfectly with theoretical model (Table 18). This will be discussed more fully in a later section (Section H).

In view of the different results found with Nuclepore and Millipore membranes, the most plausible solution to the unusual kinetic behavior observed in Nuclepore has to be deduced from the different physico-chemical nature of Nuclepore and Millipore. Besides the clews, the most remarked difference between these two types of membrane is their molecular composition; that is, the Nuclepore membrane is a polycarbonate and the Millipore membranes are cellulose esters.

In his extensive review on the membrane transport, Lakshminarayanaiah (23) came to the conclusion that functionally, no membrane exists which is completely inactive when used as a barrier to separate two solutions, unless it is too porous (e.g., 80% porosity in Millipore membranes). Autian (58) published two review articles on the polymeric membranes and stated that the chemical structure of both the penetrant species and the polymer are the determining factors in the types of physico-chemical interactions that can happen between them. A description of the Nuclepore membranes on a molecular scale indicates the following: Nuclepore is made from polycarbonate materials prepared
synthetically from the monomer—bisphenol-A, \((2,2\text{-bis (4-hydroxyphenol)propane})\) \((92, 101, 102)\). The structural formula for the bisphenol-A polycarbonate may be depicted as

\[
\begin{align*}
\text{H} & \quad \text{O} \quad \text{CH}_3 \\
\text{C} & \quad \text{O} \quad \text{C} \\
\text{CH}_3 & \quad \text{O} \quad \text{C} \\
& \quad \text{n}
\end{align*}
\]

Because of the high degree of aromaticity in both polycarbonate polymers and Nitrophenol molecules we may expect that some types of weak physical interaction, e.g., hydrophobic interaction between these aromatic \(\Pi\)-electron systems, may occur between Nitrophenol molecules and Nucleopore membranes. The nucleation-damaged tracks in polycarbonate membranes are composed of end-group species which are more highly reactive than the bulk polymer and as a result have greater chemical activity \((101, 103)\). Therefore, in their transport through the pore channels in the membrane structure, Nitrophenol molecules may react with bulk polymer or the active end-groups of polycarbonate polymer chain in some way. Thus, the rate of membrane transport may be decreased and deviations arise from the values expected from theoretical calculation using the literature diffusivity.
The degree of deviation, \((k_m)_E/(k_m)_T\), of the kinetics of membrane transport is further analyzed in Figure 26. This plot indicates that both para- and ortho-Nitrophenols show the same type of restrictive effect due to the pore size of Nuclepore membranes but that the degree of restriction is slightly larger for ortho- than for the para-isomer. This observation coincides with the behavior reported in Table 13 concerned with the effect of membrane thickness. The higher degree of deviation from theory demonstrated by ortho-Nitrophenol may be explainable on the basis of its intramolecular hydrogen bonding and the weaker solvent-solute interaction it exhibits (66). The intermolecular hydrogen bonding found in para-Nitrophenol will result in a stronger solvent-solute interaction which may oppose the sorption process of para-Nitrophenol to the polycarbonate polymers.

Since only a small disc of Nuclepore membrane (0.23 cm\(^2\)) is used in the membrane permeation studies, the effect of drug-membrane interaction does not reduce the bulk concentration enough to show up in the steady-state permeability measurements.

Therefore, it is obvious that, from the results obtained that the mechanism of membrane permeation at the non-steady-state may be quite different from that predicted by diffusion theory. The present investigations clearly demonstrate that the results on membrane permeation at steady-
Figure 26 Effect of the pore size of Nuclepore membrane on the kinetics of membrane transport of Nitrophenols (5.56x10^{-5} M; pH 4.0; 30°C): ● para-
◯ ortho-isomers.
state can not be applied to extrapolate or interpret the mechanism of membrane transport at non-steady-state. Both must be disclosed and treated simultaneously for a real understanding of the nature of membrane permeation of drug. On the other hand, the theoretical reality of Eqs. (16-2) and (18-2), which describe the dependency of $k_m$ and $P_m$ on the magnitude of $(\delta_D + \delta_m)$, was also investigated. For this purpose, the experimental data are plotted according to Eqs. (26) and (27) and given in Figure 27. It is seen that the experimental slopes are found to be close to the theoretical slopes of -2 and -1 respectively.

The investigation on the effect of membrane thickness on membrane permeation is also extended to Dialysis membranes using the technique of consecutive steady-state. Results are shown in Table 15. The gross results observed in Nuclepore membranes also are found to occur here. The longer the diffusional path, the lower the $(k_m)_E$ and $(P_m)_E$ values become. The reasons for this behavior may be more akin to the results obtained with Millipore membranes as described later.

Because no information on porosity is available from the manufacturer, we can not make any further analysis of the degree of deviation of the experimental values from their respective theoretical data. However, it is possible for us to make a comparison between these two membrane thicknesses. That is : when the value of $(\delta_D + \delta_m)$ increases
Figure 27  $P_m$ and $k_m$ of p-Nitrophenol ($5.56 \sim 15.0 \times 10^{-5}$M; pH 4.0; 30°C) across Nuclepore membrane (11.7~58.5x10^{-4} cm) as a function of thickness. The respective slopes are $\bigcirc$ - 0.97 and $\Box$ - 1.88 as compared to theoretical values of -1.0 & -2.0.
TABLE 15

THE PERMEATION OF p-NITROPHENOL THROUGH DIALYSIS MEMBRANES AND A COMPARISON BETWEEN EXPERIMENTAL AND THEORETICAL VALUES

<table>
<thead>
<tr>
<th>$\delta_D + \delta_m$ (x10^{-4} cm)</th>
<th>Rate-constant $\frac{(k_m)_E}{(k_m)_T}$ (second^{-1})</th>
<th>Membrane Permeability $\frac{(P_m)_E}{(P_m)_T}$ (x10^{-4} µa/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.37</td>
<td>6.154 0.215 0.035</td>
<td>21.15 4.43 0.21</td>
</tr>
<tr>
<td>66.13</td>
<td>2.072 0.027 0.013</td>
<td>12.27 1.30 0.106</td>
</tr>
</tbody>
</table>

* $\delta_D = 18.21 \times 10^{-4}$ cm at a rotation speed of 1200 rpm.
1.72-fold from $38.37 \times 10^{-4}$ cm to $66.13 \times 10^{-4}$ cm, then, theoretically, the $(P_m)_E/(P_m)_T$ and $(k_m)_E/(k_m)_T$ data should decrease in a magnitude of 1.72-fold and 2.96-fold respectively if theoretical models of Eqs. (16) and (18) are obeyed. Experimentally, it is seen that the $(P_m)_E/(P_m)_T$ is reduced 1.98-fold and the $(k_m)_E/(k_m)_T$ is lowered 2.66-fold. Obviously, the experimental results are very close to those expected from theoretical calculation with a deviation of 10-15%. Due to the uncertainty about the porosity and swelling of Dialysis membranes reported in literature (57), we cannot more closely discuss membrane transport mechanisms in this type of membrane.

It is also interesting to note that the porosity of Dialysis membranes determined by steady-state membrane permeability was found to agree perfectly with the value estimated by the semi-infinite linear diffusion model (Eq. 25) (Figure 16).
H. Tortuosity in Millipore and membrane permeation

Previously, it was pointed out that Millipore membrane is a good model membrane for the investigation of the influence of the membrane pore size on the membrane permeation since a wide range of pore sizes are available that have the same porosity (80%) and a constant thickness ($150 \times 10^{-4}$ cm). The application of "Single Steady-state Technique" demonstrated that the membrane permeation of Nitrophenol was limited by the pore size of Millipore membrane (Table 10) as expected from literature reports (30-35). Using the new methodology, "Consecutive Steady-state Technique", the effect of membrane pore size on both the steady-state membrane permeability and the dynamics of membrane transport was studied.

For all the Millipore membranes studied, the linear relationship of diffusion current to bulk drug concentration in donor compartment is perfectly obeyed. Figure 28 gives three typical sets of data. The slope of these straight lines is defined as the steady-state membrane permeability (Eq. 18). The values of steady-state membrane permeability ($P_m$) decrease as the pore size of the Millipore membrane decreases; in this instance, from type SM ($5 \times 10^{-4}$ cm) to type GS ($2.2 \times 10^{-5}$ cm) (Figure 28).
Figure 28 Relationship between diffusion current at steady-state ($i_{ss}$) and concentration of p-Nitrophenol ($C_d$) in a Millipore membrane (thickness: 150 microns; porosity: 80%). The resultant steady-state membrane permeability decreases as the pore size of membrane decreases:

- SM (5x10$^{-4}$ cm pore) 3.26x10$^5$ μA/M
- RA (1.2x10$^{-4}$ cm pore) 2.1x10$^5$ μA/M
- GS (0.22x10$^{-4}$ cm pore) 0.51x10$^5$ μA/M
Millipore membranes are 150 x 10^{-4} cm thick, in the membranes of small pore size (< 0.22 microns) the time required to reach the real steady-state is much longer than that in the membranes of larger pore size (> 0.45 microns) or in the thinner membranes such as Nuclepores. So, the Viale analysis (79), grounded on the Guggenheim treatment (78) for first-order kinetics where a long time course is needed to reach an equilibrium state, is adapted to treat the kinetic data obtained with Millipore membranes. The rate-constants for membrane transport, $k_m$, obtained by Viale analysis have been found to be close to those estimated by First-order kinetic plot with a variation of ± 10%. Figure 29 is a comparison between the Viale analysis ($t (i_{t+\Delta} - i_t)$ vs. time) and the First-order kinetic plot ($\log(i_{SS} - i_t)$ vs. time) where, in the latter, $i_{SS}$ is estimated.

As in the case of steady-state membrane permeability, the rate-constants for membrane transport ($k_m$) are also considerably affected by the pore sizes of Millipore membrane. The data in Table 16 demonstrates that the smaller the pore size or the single pore area of the membrane, the lower the magnitude of $k_m$ and $P_m$. This relationship coincides with the observations of Uzelac and Cussler (30), Ullah and Cadwallader (31) and Manegold (32-35) on the effect of membrane pore size.
Figure 29 Agreement between the rate-constant for membrane transport calculated from the first-order kinetic plot \( \circ k_m = 0.0060 \text{ second}^{-1} \) and Viale analysis (Reference 79) \( \odot k_m = 0.0065 \text{ second}^{-1} \).
### Table 16

The rate constant and steady-state permeability of membrane transport as a function of pore area of the Millipore membrane

<table>
<thead>
<tr>
<th>Millipores</th>
<th>Single Pore Area $(10^{10} \text{cm}^2)$</th>
<th>$(k_m)_E$ $(\text{second}^{-1})$</th>
<th>$(P_m)_E$ $(10^{-5} \mu \text{A}/\text{M})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>5026.6</td>
<td>0.3333</td>
<td>3.89</td>
</tr>
<tr>
<td>SM</td>
<td>1963.5</td>
<td>0.2222</td>
<td>3.26</td>
</tr>
<tr>
<td>SS</td>
<td>706.9</td>
<td>0.1190</td>
<td>2.52</td>
</tr>
<tr>
<td>RA</td>
<td>131.1</td>
<td>0.0952</td>
<td>2.18</td>
</tr>
<tr>
<td>GS</td>
<td>3.8</td>
<td>0.0064</td>
<td>0.50</td>
</tr>
<tr>
<td>VC</td>
<td>0.8</td>
<td>0.0042</td>
<td>0.44</td>
</tr>
<tr>
<td>VM</td>
<td>0.2</td>
<td>0.0040</td>
<td>0.42</td>
</tr>
</tbody>
</table>
The result will be even more dramatic if we plot the steady-state membrane permeability ($P_m$) data and the rate-constant for membrane transport ($k_m$) data against single pore area. As shown in Figures 30 and 31, when the single pore area is smaller than approximately $5 \times 10^{-7}$ cm$^2$, both data, $P_m$ and $k_m$, show two types of behavior depending on the single pore area range. The relationship between $P_m$ and $k_m$ to single pore area are similar to that of water flow rate vs. single pore area. The data on water flow rate reported by the manufacturer of Millipore membranes also shows two types of behavior with varying single pore area (Figure 32). The similarity in the influence of membrane pore size on the water flow rate and on the membrane permeation of Nitrophenols suggest that the mechanism of membrane transport is by way of the water-filled pore channels in the membrane structure (99). It has been shown in literature (95) that the membrane permeation of phenol is coupled with water flux.

This biphasic dependency with respect to pore area suggested the involvement of another membrane variable, the tortuosity. The tortuosity may be an important parameter since the Millipore membranes are relatively thick, $150 \times 10^{-4}$ cm. There is no information regarding tortuosity from the manufacturer's specifications.
Figure 30  Effect of the area of a single pore in Millipore membrane on the steady-state membrane permeability of p-Nitrophenol (5.56~15.0x10^-5M; pH 4.0; 30°C).

Upper curve: Large pore (>1.3 x 10^-8 cm²)
Lower curve: Small pore (<3.8 x 10^-10 cm²)
Figure 31 Effect of the area of a single pore in Millipore membrane on the dynamics of membrane transport of p-Nitrophenol ($5.56 \times 10^{-5}$M; pH 4.0; 30°C).

Upper curve: Large pore ($>1.3 \times 10^{-8}$ cm²)
Lower curve: Small pore ($<3.8 \times 10^{-10}$ cm²)
Figure 32 Effect of the area of a single pore in Millipore membrane on the water flow rate whose data were extracted from manufacturer's literature (with a pressure differential of 70 cm of mercury).

Upper curve: Large pore ($>1.3 \times 10^{-8}$ cm$^2$)
Lower curve: Small pore ($<1.6 \times 10^{-9}$ cm$^2$)
The steady-state membrane permeability data compiled in the studies consists of at least six determinations of steady-state diffusion current per unit concentration ($i_{ss}/\text{conc.}$). Because it represents steady-state measurements, we consider it more accurate in the study of tortuosity effects in Millipore membranes assuming that tortuosity is important. The last column in Table 17 lists the assumed tortuosity calculated by taking the ratio of the experimental $P_m$ data over the corresponding theoretical $P_m$ which was computed from

$$
(P_m)_T = \frac{nF\epsilon D}{(\delta_D + J\cdot\delta_m)}
$$

(48)

where $J$ is the tortuosity of Millipore membranes.

The pore sizes of the Millipore membranes listed in Table 17 are at their smallest 100-fold more than the largest molecular diameter of the Nitrophenols (assuming they are spherical species) and hence should show normal diffusion (29). In addition, the membranes have a constant porosity and thickness. Assuming that tortuosity is causing the discrepancy between theoretical and experimental values, we calculated tortuosity from its steady-state data and applied its calculated value to kinetic data to see if a better fit to experimental rates would be attained. It is evident from the data of Table 18 that this concept is supported.
<table>
<thead>
<tr>
<th>Millipores</th>
<th>Pore Size (x10^{-4} cm)</th>
<th>$P_m$ (x10^5 \mu a/cm)</th>
<th>Tortuosity $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>8.00</td>
<td>4.515</td>
<td>3.89</td>
</tr>
<tr>
<td>SM</td>
<td>5.00</td>
<td>5.125</td>
<td>3.26</td>
</tr>
<tr>
<td>SS</td>
<td>3.00</td>
<td>4.435</td>
<td>2.52</td>
</tr>
<tr>
<td>RA</td>
<td>1.20</td>
<td>4.381</td>
<td>2.18</td>
</tr>
<tr>
<td>GS</td>
<td>0.22</td>
<td>4.419</td>
<td>0.50</td>
</tr>
<tr>
<td>VC</td>
<td>0.10</td>
<td>4.515</td>
<td>0.44</td>
</tr>
<tr>
<td>VM</td>
<td>0.05</td>
<td>4.393</td>
<td>0.42</td>
</tr>
</tbody>
</table>

$^a$ Theoretical values of steady-state permeability are calculated from Eq. (46).

$^b$ Tortuosity data are calculated from

$$
\frac{(P_m)_E}{(P_m)_T} = \frac{(\delta_p + \delta_m)}{(\delta_p + \delta_m)}
$$
<table>
<thead>
<tr>
<th>Millipore</th>
<th>$k_m$</th>
<th>(second$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
<td>Experimental</td>
</tr>
<tr>
<td>SC</td>
<td>0.3381</td>
<td>0.3333</td>
</tr>
<tr>
<td>SM</td>
<td>0.1844</td>
<td>0.2222</td>
</tr>
<tr>
<td>SS</td>
<td>0.1128</td>
<td>0.1190</td>
</tr>
<tr>
<td>RA</td>
<td>0.0865</td>
<td>0.0952</td>
</tr>
<tr>
<td>GS</td>
<td>0.0054</td>
<td>0.0064</td>
</tr>
<tr>
<td>VC</td>
<td>0.0043</td>
<td>0.0042</td>
</tr>
<tr>
<td>VM</td>
<td>0.0042</td>
<td>0.0040</td>
</tr>
</tbody>
</table>

* Theoretical values of rate-constant for membrane transport are calculated from Eq. (49) where the tortuosity calculated from steady-state permeability data is included to account for the increase in the effective length of pore channels in the membrane due to tortuosity.
The tortuosity data computed and given in Table 17 are applied to the following theoretical rate constant equation to estimate the theoretical values of the rate-constant for membrane transport, $(k_m)_T$:

$$(k_m)_T = \frac{\Pi^2 D}{(\delta_D + J \cdot \Sigma_m)^2} \quad (49)$$

The calculated values of $(k_m)_T$ are tabulated in Table 18 together with the experimentally observed values of the rate-constant for membrane transport $(k_m)$. Comparing these two sets of data shows that by taking the tortuosity into account a perfect agreement between the theoretical and the experimental values of $k_m$ exists. This agreement strongly demonstrates the involvement of membrane tortuosity in both non-steady-state and steady-state membrane permeations. In non-steady-state, the rate of membrane transport is inversely proportional to membrane tortuosity as expressed in Eq. (49); and at steady-state, the steady-state membrane permeability depends on the reciprocal of the membrane tortuosity as defined in Eq. (48).

It should be noted in Table 17 that the tortuosities in the membranes of pore size smaller than $2.2 \times 10^{-5}$ cm are much larger than those in the membranes of pore size larger than $1.2 \times 10^{-4}$ cm. This difference in the magnitude of tortuosity just coincides with the transition in the relation between $P_m$, $k_m$ and water flow rate to single pore
area (Figures 30-32 and Table 17).

According to the theoretical analysis of Higuchi and Higuchi (36) on the diffusion through heterogeneous barriers, both the steady-state transport and non-steady-state diffusion would be expected to be inversely proportional to tortuosity. Higuchi and his associates (80, 81) have made a study of drug release from wax matrices. Based on their arguments, the membrane tortuosity values can be as small as slightly more than 1.0 and as large as greater than 1000 depending on the physico-chemical properties and the composition of the matrix. Their tortuosity data on salicylic acid and benzoic acid are relatively constant and are 12.8 to 16.9 and 14.7 to 18.9, respectively. Furthermore, another studies have recently been done by Farhadieh et al. (37, 82) on drug release from the matrix of methylacrylate-methylmethacrylate copolymer. They observed that the tortuosity in the polymer matrix can have values from 6.3 to 37 depending on the drug species. They also found that the tortuosity can be greatly increased by exposure of polymer matrix to acetone vapor resulting in the reduction of the rate of drug-release. Based on these reports (36, 37, 80-82), the tortuosity data calculated in the present studies are very reasonable in their magnitude.

It was reported earlier in Section (G) that the observed decrease in the rate-constant for membrane transport ($k_m$) of Nitrophenol isomers across Nuclepore membrane might
be due to the possible drug-polymer interactions of aromatic \( \pi \)-electron systems. Millipore membranes are composed of mixtures of cellulose nitrate and cellulose acetate (94). The three active hydroxyl groups in each glucosan unit of the linear \( \alpha \)-cellulose polymer chains are esterified to form acetate or nitrate (57). The esterification of these active hydroxyl groups produces a marked difference in the physico-chemical properties of cellulose membrane (58), for instance, the hydrophilic character and drug-membrane interaction nature are remarkably altered. On this basis, we would not expect a Millipore-Nitrophenol interaction.

Tuwiner (57) concluded that if a membrane is considered to be an inert physical barrier between two solutions, the coefficient of transfer for a given membrane material should be proportional to the diffusivities of the penetrant species in the solution and inversely function of the thickness of the barrier. Up to now, a number of experimental results done in the present study have provided strong evidence for the eligibility of Eqs. (48) and (49) in expressing the mechanisms of membrane permeation at steady-state and at non-steady-state transport. Both these Equations fall in the criteria Tuwiner set for an inert membrane. So, in the present studies, we may conclude that the tortuosity effect is the primary parameter responsible for the decrease in both the rate and the amount of membrane permeation through Millipore membranes. On the other hand,
no tortuosity or a negligibly small one, if any, may be present in Nuclepore membrane as is seen in the steady-state permeability data (Tables 12 and 14) since the experimental values of steady-state membrane permeability are equal to those of theoretical data after the manufacturer's listed porosity is taken into account. This result is in agreement with the manufacturer's information about Nuclepore membrane: The pores in the membrane are uniform, smooth-bore, round holes which pass essentially straight through the membrane.
# APPLICATIONS

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</tbody>
</table>
A. Introduction

Classical methods, employed to detect, determine and study the binding characteristics of small molecules to serum albumin, such as equilibrium dialysis, ultrafiltration, ultracentrifugation and electrophoresis have been discussed by a number of authors (104-106). Several years ago, Stein (107) reported a rapid, non-equilibrium dialysis method which was shown to yield, for the binding of methyl orange to protein, results which are comparable to those obtained by equilibrium methods. Recently, Meyer and Guttman (108-110) devised a new methodology for protein binding studies, which is based on the fact that the rate of disappearance of small drug molecules from a dialysis sac with protein is proportional to the concentration of the unbound species.

Differences in the polarographic behavior of bound and free substrates have been demonstrated and applied to the determination of binding parameters of Evans blue by Markus and Baumberger (111). One year before this, Saroff and Mark (112) designed an experimental method, which was based on the combination of amperometric titration and dialysis experiments, to analyze polarographically the complexes of Serum Albumin-Mercury and of Serum Albumin-Zinc.

Sokoloski and his associate (113) have recently utilized the techniques of equilibrium dialysis, spectrophotome-
tric determination and extrinsic rotatory dispersion to investigate the competitive binding of 2-(4'-hydroxybenzene azo)benzoic acid (HBABA) and \( \alpha-(4\text{-Chlorophenoxyl})\alpha\text{-methylpropionic} \) acid (CPMPA) with a number of serum albumin preparations obtained from different species. Their results revealed an unique behavior for the rat serum albumin (RSA) and suggested that CPMPA is able to cause a small molecular perturbation in the RSA which may liberate additional sites for HBABA binding.

In the previous sections, we have discussed the function of rotating-disc electrode polarographic instrumentation, the usefulness of consecutive steady-state technique, and their successful application in the studies of membrane permeation. The present investigation intends to utilize the knowledge gained in these previous studies to study the interaction of drug molecules with protein. The intent was to utilize the methodology developed to conveniently characterize drug-protein binding in very short time periods.
B. Theory

Most investigators have been concerned with protein-small molecule interaction at equilibrium and with the determination of degree of binding as a function of compositional and environmental variables.

A protein molecule is composed of multiple binding sites which may be electrostatic or hydrophobic in nature. Thus, multiple equilibria exist in the protein-drug binding process:

\[
P + D = PD \quad K_1 = \frac{(PD)}{(P)(D)} \quad (50)
\]

\[
PD + D = PD_2 \quad K_2 = \frac{(PD_2)}{(PD)(D)} \quad (51)
\]

\[
\vdots
\]

\[
PD_{i-1} + D = PD_i \quad K_i = \frac{(PD_i)}{(PD_{i-1})(D)} \quad (52)
\]

\[
\vdots
\]

\[
PD_{n-1} + D = PD_n \quad K_n = \frac{(PD_n)}{(PD_{n-1})(D)} \quad (53)
\]

where \( P \) represents free protein, i.e., Bovine Serum Albumin; \( D \) free drug, i.e., HBABA; and \( PD_i \) the protein-drug complex in which \( i \) molecules of drug species are bound to one protein molecule; \( n \) is the maximum number of drug mole-
cules which can be bound to one molecule of protein.

A number of methods for treating and presenting the results of such multiple equilibria are commonly used. The Scatchard plot of experimental data is employed frequently and is directly derived from mass-law considerations. It can be shown (105) from such considerations that reversible binding of drug molecules to protein is described by the following equation where more than one class of sites are possible:

\[ r = \sum_{i=1}^{i} \frac{n_iK_iD}{1+K_iD} \]  \hspace{1cm} (54)

where \( r \) is the moles of drug molecules bound to total moles of protein in the multiple equilibrium system; \( n_i \) is the number of binding sites in the \( i \)th class of sites; \( K_i \) the intrinsic association constant for the binding of drug molecules by sites in the \( i \)th class and \( D \) the concentration of unbound drug molecules.

Equation (54) assumes that activities can be represented by concentrations in dilute solution, that all sites within a class are equivalent in binding affinity, and that all sites are mutually independent. For a single class of sites:

\[ r = \frac{nKD}{1 + KD} \]  \hspace{1cm} (55)
Equation (55) can be rearranged to yield (Scatchard treatment):

\[
\frac{r}{D} = nK - rK
\]  

(56)

\(r/D\) is a linear function of \(r\); extrapolation to abscissa and ordinate allows estimation of \(n\) and \(nK\). Curvature in such plots is assumed to indicate the existence of more than one class of sites, i.e.,

\[
r = \frac{n_1K_1D}{1+K_1D} + \frac{n_2K_2D}{1+K_2D} + \ldots + \frac{n_jK_jD}{1+K_jD}
\]  

(57)

Graphical treatment of experimental binding data by means of the Scatchard plot does not heavily weight those experimental points which are obtained at low concentration of free drug and will not, therefore, lead to misinterpretations concerning the binding behavior at high concentration of free drug. Hence, the Scatchard treatment in pharmaceutical laboratories is the graphical method of choice.

In the present investigations, the system is so designed that a sheet of thin membrane, whose pore size is so small that only the small molecules of free drug are membrane-permeable (protein as well as the drug molecules bound to the protein are impermeable). The membrane is positioned on the immediate surface of the rotating-disc electrode as described in the previous section on membrane permeation.
In this system, therefore, the diffusion current, $i_{ss}$, at steady-state is related to the free drug concentration as follows:

$$i_{ss} = P_m (D)_f$$

(58)

where as before $P_m$ is defined as the steady-state membrane permeability. Thus, the concentration of free drug, $(D)_f$, can be described as

$$(D)_f = \frac{i_{ss}}{P_m}$$

(59)

When protein is present in the system, the concentration of free drug is expected to decrease since a fraction of it is bound to the protein and thus becomes membrane impermeable. Therefore, the concentration which is bound, $(D)_b$, to the protein molecules can be calculated from the concentration of free drug in the presence and absence of protein molecules as follows:

$$(D)_b = (D)_o - (D)_p$$

(60)

where $(D)_o$ is the concentration of free drug in the absence of protein molecules and $(D)_p$ is the concentration in the presence of protein. The binding ratio, $r$, can be expressed as

$$r = \frac{(D)_b}{(P)}$$

(61)
The binding parameters of a given drug species to a particular protein molecule can be easily estimated from membrane permeation measurements of free drug in the presence and absence of protein species on the basis of Eqs. (59), (60) and (61). These binding data are then subjected to Scatchard analysis and with the aid of a computer which has been programmed based on Eq. (57) (assuming two sites exist), estimates of the number of binding sites in the $i$th class of sites ($n_i$) and the intrinsic association constant for the binding of drug molecule by sites in the $i$th class ($K_i$) can be made.
C. Methodology

(1) Reagents and Materials:

(a) Bovine Serum Albumin (BSA)

Two brands of BSA were tested: (i) Bovine Albumin (Fraction V powder) was purchased from Pentex Incorporated, Kankakee, Illinois 60901 for some preliminary binding studies using "Single Steady-state Technique", and (ii) Albumin Bovine (Fraction V powder) fatty acid poor was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio 44128 for the binding studies using "Consecutive Steady-state Technique" and the competitive binding studies using both techniques.

(b) Rat Serum Albumin (RSA)

Rat Albumin (Fraction V powder) from Pentex was used for the competitive binding studies.

(c) Human Serum Albumin (HSA)

Human Serum Albumin (Fraction V powder) from Nutritional Biochemicals Corp. was used in the Nitrophenol binding studies.

(d) 2-(4'-Hydroxy Benzene Azo) Benzoic Acid (HBABA)

Two brands of HBABA were purchased from: (i) Eastman Organic Chemicals Co., Rochester 3, New York; and (ii) Nutritional Biochemical Corp., Cleveland, Ohio 44128.
Both of these products, which have the molecular structure as shown, have been subjected to polarographic analysis for electrochemical properties.

\[
\text{HO} \quad \text{N} = \text{N} \quad \text{COOH}
\]

(e) \(\alpha\)-(4-Chlorophenoxy)\(\alpha\)-methylpropionic Acid (CPMPA)

This material as the ester has been marketed as an anti-hypercholesterolemia agent for many years and was supplied through the courtesy of Dr. D.T. Witiak of this laboratory (Division of Medicinal Chemistry). The molecular structure of CPMPA is:

\[
\text{CH}_3
\]
\[
\text{Cl} \quad \text{O} \quad \text{C} \quad \text{COOH}
\]
\[
\text{CH}_3
\]

(f) Buffered supporting electrolyte system

The phosphate buffer solution of pH 7.4 described earlier in the membrane permeation studies was used to prepare the solutions of Serum Albumin, of HBABA, and of CPMPA.
(g) **Dialysis tubing membrane**

Dialysis tubing, "1 7/8 s.s. dialysis" type, with an average pore radius of 24 Å and a thickness of 0.0016 inch purchased from Union Carbide Corp., Chicago, Illinois 60638, was utilized to separate the free drug species from the bound drug and to prevent contamination of the electrode surface by the protein.

A disc of 0.37 inches diameter was cut from a fresh batch of Dialysis tubing and washed several times with double distilled water (boiled) and then kept in a preserving solution containing 0.3 % Benzoic acid and 10 % Glycerine.

Before a scheduled experiment, the membrane disc was washed several times with double distilled water and then soaked in a refrigerated phosphate buffer overnight. Just before the measurement, the buffer-saturated membrane disc was positioned on the immediate surface of rotating-disc electrode as described previously in the section dealing with the membrane permeation studies.

(2) **Technology for measurements**

The techniques used in the present protein - drug interaction studies are essentially the same as those in the membrane permeation investigations. Both techniques, "Consecutive Steady-state" and "Single Steady
-state", have been applied satisfactorily in the present binding studies with small modification in procedure to handle the high foaming tendency of protein solutions.

(a) **Single Steady-state Technique**

Stock solutions of HBABA, of CPMPA and of protein were made separately in pH 7.4 phosphate buffer system. The mixtures were prepared from these stock solutions as follows:

(i) In the case of HBABA-protein binding studies, a series of mixtures were prepared containing a fixed concentration of protein and increasing concentrations of HBABA. After mixing, these HBABA-protein mixtures are kept in a refrigerator for a time sufficient to allow attainment of equilibrium. Before the measurements, the mixture was deaerated in the De-aeration Column (Figure 4) as previously described. Then, 20 ml. of this mixture was delivered anaerobically to the pre-deaerated righthand half-cell of polarographic cell. Finally, the same procedure as used in the membrane permeation studies was used here to measure the diffusion current of free drug.
(ii) In the case of HBABA–CPMPA–protein competitive binding studies, a series of mixtures, containing a fixed concentration of protein, a fixed concentration of HBABA, and increasing concentrations of CPMPA, were prepared. Then, the same precautions and processes as described above were followed to measure the diffusion current of free HBABA.

(b) Consecutive Steady-state Technique

Stock solutions of HBABA, of CPMPA and of protein were made separately in pH 7.4 Phosphate buffer system and deaerated individually in the Deaeration Column (Figure 4). 15 ml. of deaerated protein solution was delivered anaerobically to the pre-deaerated righthand half-cell of polarographic cell. Then, in the case of HBABA–protein binding studies, the same procedures and precautions as those described earlier for membrane permeation studies were followed; and in the case of HBABA–CPMPA–protein competitive binding studies, 1.0 ml. of HBABA was added before the consecutive addition of CPMPA solution to the mixture of HBABA–protein.

A rotation speed of 1200 r.p.m. was used in all the drug–protein binding studies in order to prevent the formation of foam in the system containing protein substances.
D. Results and Discussions

(1) Electrochemical reduction:

In studies with a dropping-mercury electrode (DME), the potassium ion in the phosphate buffer solution (pH 7.4) was observed to have a decomposition potential \((E_d)\) of \(-1.74\) volts prior to which a wide range of potential having a small residual current could be utilized in the present studies.

Experimentally, HBABA may be reduced electrochemically as follows with the consumption of two electrons:

\[
\begin{align*}
\text{HO} & \quad \text{N} = \text{N} & \quad \text{GOOH} \\
\text{GOOH} & \quad 2 \frac{(H)}{e^-} & \quad \text{HO} \quad \text{N} = \text{N} & \quad \text{GOOH}
\end{align*}
\]

As demonstrated in Table 19, both HBABA and BSA are electrochemically reducible. Two reduction waves at half-wave potentials of \(-0.1\) and \(-0.94\) volt are observed for BSA where the decomposition potential \((E_d)\) occurs at \(-0.01\) volt.

HBABA is reduced electrochemically with the formation of one two-electron wave having a decomposition potential of \(-0.14\) volt and a half-wave potential of \(-0.30\) volt. When a mixture of HBABA and BSA is subject to polarographic studies, a more complicated
<table>
<thead>
<tr>
<th>Solution*</th>
<th>Electrochemical Character (volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-$E_d$</td>
</tr>
<tr>
<td></td>
<td>1st wave</td>
</tr>
<tr>
<td>BSA</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>HBABA</td>
<td>0.14</td>
</tr>
<tr>
<td>BSA</td>
<td>0.15</td>
</tr>
<tr>
<td>HBABA</td>
<td>5</td>
</tr>
</tbody>
</table>

* 6 x 10^{-5} M of BSA and/or 30 x 10^{-5} M of HBABA in pH 7.4 phosphate buffer (0.1 M) which has $E_d$ of -1.74 volt and $E_{1/2}$ in a negative potential range beyond the plateau potential of 2nd wave.
result was observed. As shown in Table 19, the first wave at -0.1 volt in BSA disappears and is overlapped by the reduction wave of HBABA at -0.29 volt while the second wave at -0.94 volt remains unchanged. $E_d$ also shifts from -0.01 volt to -0.15 volt. It was also noted that the current height at plateau region for both BSA waves were increased due to the addition of HBABA (Table 20). The fact that the reduction waves of both HBABA and BSA makes the measurement of free HBABA concentration and, in turn, the estimation of binding parameters difficult and complicated, since there is no way to know that the HBABA bound to protein is electrochemically inactive.

When a rotating-disc carbon electrode is used for investigation, both the $E_d$ and $E_h$ values for HBABA are shifted to a more negative potential (Table 21) as noted earlier in the case of Nitrophenols (Tables 1 & 2). Interestingly, two reduction waves, each of them a one-electron process, were observed for HBABA at -0.41 and -0.60 volts respectively.

Experimentally, CPMPA was observed to be electrochemically inactive and would not influence the electrochemical behavior of HBABA (Table 21). This result indicates that the reduction of free HBABA will be the only contributor to the current in mixtures of HBABA, CPMPA and BSA with a membrane covering the
TABLE 20

BINDING BETWEEN HBABA AND BSA STUDIED WITH DROPPING MERCURY ELECTRODE

<table>
<thead>
<tr>
<th>Solution*</th>
<th>Conc. (x10^{-5} M)</th>
<th>Limiting Current at Plateau</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First wave at E_p = -0.50 V</td>
</tr>
<tr>
<td>BSA</td>
<td>12</td>
<td>0.37 µa</td>
</tr>
<tr>
<td>HBABA</td>
<td>60</td>
<td>3.79 µa</td>
</tr>
<tr>
<td>BSA</td>
<td>12</td>
<td>2.11 µa</td>
</tr>
<tr>
<td>HBABA</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

* Viscosity difference between HBABA, BSA and HBABA+BSA in K-phosphate buffer (pH 7.4) is only 4% at 24°C.
### TABLE 21

**EFFECT OF CPMPA ON THE ELECTROCHEMICAL PROPERTIES OF HBABA**

<table>
<thead>
<tr>
<th>Species</th>
<th>Conc. ( \times 10^4 \text{ M} )</th>
<th>Electrochemical Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( i_{ss}/\text{conc.} ) ( \times 10^{-4} \text{mA/M} )</td>
<td>( -E_d ) (volt)</td>
</tr>
<tr>
<td>CPMPA</td>
<td>60</td>
<td>1.04(^b)</td>
</tr>
<tr>
<td>HBABA</td>
<td>4</td>
<td>25.8</td>
</tr>
<tr>
<td>CPMPA</td>
<td>60</td>
<td>26.1</td>
</tr>
<tr>
<td>HBABA</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

\( a \)  
In K-phosphate buffer (pH 7.4) and at 30°C.

\( b \)  
The electrochemical properties of phosphate buffer are same with and without CPMPA.
electrode and that the change in the flux of free HBABA is a valid indicator in the competition of CPMPA and HBABA for binding sites on BSA.

The difference in viscosity among HBABA, BSA and HBABA-BSA mixture was found to be as small as 4%. Therefore, any viscosity effect on electrochemical properties may be expected to be negligibly small.

(2) Effect of covering membrane:

When a constant potential, i.e., -1.00 volt, is applied to the system, the diffusion current due to the electroreduction of HBABA is found to be a linear function of the HBABA concentration in the bulk of donor solution compartment (Figure 33). The permeability calculated from the slope is found to be $1.5 \times 10^5 \, \mu A/M$. When a sheet of Dialysis membrane covers this rotating-disc electrode, the linear relationship between diffusion current and concentration is still obeyed although the slope is reduced. The permeability ratio, i.e., the ratio of permeability with membrane to that without membrane, is found to be 4.2\% (Figure 33).

It is reported in literature that, in addition to the deformation of polarographic waves, the protein also can influence the electrode process by adsorption on electrode surface. This surface adsorption
Figure 33  Relation between steady-state diffusion current and concentration of HBABA.

- O : Naked electrode, $P_m = 150 \times 10^3 \mu a/M$.
- ☼ : Dialysis membrane covering electrode $P_m = 6.3 \times 10^3 \mu a/M$. 
results in low reproducibility and a protein coated electrode which can not be used again without cleaning. This fact initiates the desirability of protecting the electrode surface from contamination by protein by covering the electrode with a membrane sheet as in the present system. This membrane sheet should contain pores whose pore size is so small that only water molecules and drug molecules can go through, and one where the protein molecules and the drug molecules bound to the protein become membrane impermeable. Such a membrane is provided by Dialysis tubing which is frequently used in equilibrium Dialysis techniques and hence also used in the present investigations. It is important to compare the binding characters obtained with a covering membrane to that without a covering membrane. The comparison made in Table 22 demonstrates that essentially the same binding behavior is obtained using the "Rotating-disc Electrode" with covering Dialysis membrane.

Thus, the use of a covering membrane gives several advantages: (i) the concentration of HBAABA molecules transported is so small that the equilibrium between free HBAABA and HBAABA-ESA complex will not be significantly influenced; (ii) the electrode surface will not be coated by a film of protein (121), so the sensitivity of measurement will not be reduced and the
### TABLE 22

**EFFECT OF MEMBRANE COVERING ON PROTEIN BINDING BEHAVIOR OF HBABA TO BSA**

<table>
<thead>
<tr>
<th>Barrier&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein Binding Data&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(D)&lt;sub&gt;f&lt;/sub&gt; (x10&lt;sup&gt;-4&lt;/sup&gt;M)</th>
<th>(D)&lt;sub&gt;b&lt;/sub&gt; (x10&lt;sup&gt;-4&lt;/sup&gt;M)</th>
<th>r</th>
<th>r/(D)&lt;sub&gt;f&lt;/sub&gt; (x 10&lt;sup&gt;-4&lt;/sup&gt;/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without</td>
<td></td>
<td>0.48</td>
<td>1.92</td>
<td>1.60</td>
<td>3.33</td>
</tr>
<tr>
<td>With</td>
<td></td>
<td>0.44</td>
<td>1.96</td>
<td>1.64</td>
<td>3.76</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dialysis membrane (0.0016 inch-thick, 24 k<sup>2</sup> pore)<br>

<sup>b</sup> 2.4 x 10<sup>-4</sup> M of HBABA and 1.2 x 10<sup>-4</sup> M BSA in pH 7.4 Phosphate buffer at 30 °C.
electrode can be repeatly used; and (iii) only the free HBABA is membrane permeable, so the magnitude of diffusion current is a function of free HBABA concentration only, no other factors complicate the linear relationship.

(3) **Protein binding studied by "Single Steady-state Technique"**: 

The relation between current and added HBABA is given in Figure 34 where a range of fixed amounts of BSA were used. The lower currents observed in the presence of BSA are due to the binding and concomitant immobility of HBABA reacted. Only free HBABA permeates the membrane and is reduced generating a current. The difference between the current in the presence and absence of BSA at a given concentration of HBABA added is a measure of the HBABA bound. Using data such as these it is possible to calculate the parameters involved in the binding of HBABA to serum albumins (Table 23).

(4) **Protein binding studied by "Consecutive Steady-state Technique"**: 

Although the more conventional "Single Steady-state Technique" gives reliable binding data, it was found to be very impractical for protein binding studies since too many solutions of drug-protein mixture
Figure 34 Effect of BSA on the concentration of free HBABA transported across the membrane as a function of BSA concentrations \(( \times 10^{-4} \text{ M} )\): \(\bigcirc 0, \bullet 1, \star 2\).
TABLE 23

BINDING DATA OF HBABA TO BSA ARE A FUNCTION OF THE CONCENTRATIONS OF BOTH HBABA AND BSA

| Concentration* (x10^4 M) | Protein Binding Data | r/(D)f (x 10^-4/M) | r |
|--------------------------|-----------------------|---------------------|---|---|
| (BSA)                    | (HBABA)               |                     |   |   |
| 0.4                      | 6                     | 1.16                | 4.75| |
|                          | 7                     | 1.05                | 5.17| |
|                          | 8                     | 1.00                | 5.70| |
|                          | 9                     | 0.89                | 5.90| |
|                          | 10                    | 0.85                | 6.33| |
| 1.0                      | 6                     | 1.39                | 3.48| |
|                          | 7                     | 1.23                | 3.86| |
|                          | 8                     | 1.13                | 4.25| |
|                          | 9                     | 1.00                | 4.50| |
|                          | 10                    | 0.94                | 4.85| |
| 2.0                      | 6                     | 2.86                | 2.40| |
|                          | 7                     | 2.49                | 2.91| |
|                          | 8                     | 2.00                | 3.20| |
|                          | 9                     | 1.71                | 3.48| |
|                          | 10                    | 1.55                | 3.78| |

* In K-phosphate buffer (pH 7.4) and at 30°C.
have to be prepared to get a complete set of binding data. In order to avoid this tedious and time-consuming procedure, the "Consecutive Steady-state Technique" well-established in the membrane permeation studies was applied to the drug-protein binding system.

It should be possible to get equally reliable binding data through the use of the "Consecutive Steady-state Technique" since the equilibrium condition should be established very rapidly and hence transport should be the rate-limiting step. This assumption that the establishment of equilibrium precedes the membrane permeation of free HBABA molecules is made on the basis of the fast rate of association of drug molecules to protein molecules that are reported in literature (114).

Experimentally, the concentration of model drugs used starts with concentrations that are smaller than the concentration of BSA in the test solution and increases to where it is several times the concentration of BSA. The volume of concentrated drug solution is small (a total of only 2 ml. in a test solution of 20 ml. is added), and the actual concentrations of both BSA and drug in the test solution after each addition of drug solution can be easily calculated since all the variables are carefully controlled.
In the use of the "Consecutive Steady-state Technique", at least 16 pairs of binding data of \( r \) and \( r/(D)f \) can be obtained in a single experimental trial continuously without sampling. These binding data are then computer analyzed (program based on Eq. 57). The computer analyzes the experimentally observed binding data, draws a theoretical line giving the best statistical fit to the experimental observations and calculates the values of \( n_i \), the number of binding sites in the \( i\)th class of sites, and \( K_i \), the intrinsic association constant for the binding of drug molecules by sites in the \( i\)th class as defined in Eq. (54) and Eq. (57) assuming 2 classes. Results are present in Figures 35, 36 and 37 for the binding of HBABA and Nitrophenol to Bovine, Rat and Human Serum Albumins. Differences in the number of binding sites in each class and in the binding affinity are observed. In general, the binding sites in the second class are more in number but weaker in affinity than those in the first class. This phenomenon is shown in all three types of Serum Albumins.

All three figures demonstrate that all experimental data fit the theoretical model of two classes of binding sites very well. This result points out the applicability of "Consecutive Steady-state Technique" and "Rotating-disc Electrode Polarography" in the
Figure 35  Scatchard plot of the binding of HBABA to BSA: \( n_1: 2.47, n_2: 123.4, K_1: 6.3 \times 10^4 \) and \( K_2: 1.07 \times 10^2 \).

○ : Experimental data

--- : Theoretical line

HBABA (9.2-83.7 x 10^{-5} M) and BSA (9.8-7.9 x 10^{-5} M) in K-phosphate buffer (pH 7.4) and at 30°C.
Figure 36  Scatchard plot of the binding of HBABA to RSA

\[ n_1: 1.65, \quad n_2: 3.98, \quad K_1: 3.07 \times 10^4 \quad \text{and} \quad K_2: 39.3 \times 10^2. \]

○ : Experimental data

--- : Theoretical line

HBABA (1-6 x 10^{-4} M) and RSA (1 x 10^{-4} M) in K-phosphate buffer (pH 7.4) and at 30°C.
Figure 37  Scatchard plot of the binding of p-Nitrophenol to HSA: $n_1 = 0.48$, $n_2 = 3.71$, $K_1 = 6.4 \times 10^4$ and $K_2 = 19.8 \times 10^2$.

○: Experimental data
—: Theoretical line

p-Nitrophenol (4-40 x 10^-5 M) and HSA (1 x 10^-4 M) in K-phosphate buffer (pH7.4) and at 30°C.
investigation of drug-protein interactions. The same binding behavior has been observed by Nazareth (117) in his binding studies between HBABA and BSA using Ultrafiltration technique and Equilibrium Dialysis technique (Figure 38). This agreement gives an additional support to the applicability of the present methodology in the protein binding study.

(5) **Effects of cationic species on the binding characters**

Experimentally, it was observed that the cationic species in the phosphate buffer system (pH 7.4) will influence the number of binding sites in each class of site and their binding affinities. A typical set of data are present in Table 24 which demonstrates that when comparing the cationic species, potassium and sodium ions, the number of binding sites for sodium ion in both classes is considerably decreased and the affinity ($K$) in first class is reduced 4-fold while in second class it is enhanced 5-fold. This result points out that differences in cationic species may yield a different ionic environment, resulting in different drug-protein interactions.

This change in protein binding characters is also reflected in the values of free energy for binding ($\Delta F^0$) (Table 24).
Figure 38 Agreement in the binding data of HBABA to BSA measured by:
- Consecutive steady-state technique
- Diafiltration
- Equilibrium Dialysis

BSA (1 x 10^{-4} M) and HBABA (3-160 x 10^{-5} M) in Na-phosphate buffer (pH 7.4) at 30°C.
TABLE 24

EFFECTS OF CATIONIC SPECIES IN PHOSPHATE BUFFER ON THE BINDING CHARACTER OF p-NITROPHENOL TO HUMAN SERUM ALBUMIN

<table>
<thead>
<tr>
<th>Binding Parametersa</th>
<th>Phosphate Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
</tr>
<tr>
<td>$N_1$</td>
<td>0.72</td>
</tr>
<tr>
<td>$N_2$</td>
<td>11.88</td>
</tr>
<tr>
<td>$K_1$</td>
<td>$26.1 \times 10^4$</td>
</tr>
<tr>
<td>$K_2$</td>
<td>$3.9 \times 10^2$</td>
</tr>
<tr>
<td>$\Delta F_1^o$ (cal/mole)$^b$</td>
<td>$7.5 \times 10^3$</td>
</tr>
<tr>
<td>$\Delta F_2^o$ (cal/mole)$^b$</td>
<td>$3.6 \times 10^3$</td>
</tr>
</tbody>
</table>

$^a$ 4 - 40 x $10^{-5}$ M of p-Nitrophenol and 1 x $10^{-4}$ M of HSA in Phosphate buffer (pH 7.4) and at 30°C.

$^b$ $\Delta F^o = -RT \ln K$
This observed difference in the binding character of drug species to protein molecules due to the presence of different cationic species may well be explained based on the flickering-cluster model of Frank and Wen (118). The net water structure-forming ion, Na⁺, will exhibit a positive hydration phenomenon (119, 120) and increase the viscosity of solution; on the other hand, the K⁺ ion exerts a net structure-breaking effect and this negative water hydration phenomenon will decrease the solution viscosity in the vicinity of interactants. The change in the viscosity of the solution phase, where the protein and drug molecules coexist, may influence the behavior or pattern of interaction between protein and drug. Thus, the decreased viscosity in the presence of K⁺ ion may enhance the binding of drug molecules to protein while in contrast, the increased viscosity in the presence of Na⁺ ion will decrease the binding of drug molecules to protein.

(6) Dynamics of membrane transport of free HBABA

When no protein is present, the membrane transport of free HBABA follows the same kinetic model (first-order rate process) as that observed with the Nitrophenols. The linear relationship of \( \log (i_{ss} - i_t) \) and time is obeyed. A representative set of data
is shown in Figure 39.

When protein exists in the solution, it was observed that the same kinetic pattern is obeyed (Figure 39), but that the rate constant for membrane transport \( (k_m) \) of free HBABA decreases from 0.0451 second\(^{-1} \) when no protein is present to 0.0319 sec.\(^{-1} \) in the presence of protein. This phenomenon is found with both the Rat and Bovine Serum Albumin.

There is general agreement that, in most system, rate of association is very rapid, for instance, the rate constant for association between albumin and two azo dyes (114) were found to be 0.36 x 10\(^6 \) and 2.1 x 10\(^6 \) mole\(^{-1} \) sec.\(^{-1} \). So, it is safe to assume that the interaction between HBABA and serum protein must happen much faster than the membrane permeation of HBABA molecules which has a rate constant for membrane transport \( (k_m) \) of 0.0451 second\(^{-1} \). Froese et al. (114) also measured the rate constants for the dissociation of their two azo dyes from BSA molecules. They are 0.35 and 2.5 second\(^{-1} \) respectively. It is possible that the dissociation rate constant of these magnitudes may affect the overall rate of membrane transport of free drug. It may be appropriate to rationalize the fact that the decrease in \( k_m \) values due to the presence of protein on the basis that the rate of membrane permeation of free HBABA may be affected by
Figure 39 First-order kinetics of membrane transport of HBABA (2.31 x 10^-2 M): ○ Without BSA and ● With BSA (9 x 10^-5 M) in K-phosphate buffer (pH 7.4), at 30°C.

The observed values of $k_m$ are ○ 0.0451 and ● 0.0319 second$^{-1}$. 
the dissociation rate of the complex.

(7) Competitive binding of CPMPA and HBABA to RSA

It was reported by Hermann (116) that with BSA, CPMPA competitively inhibited the HBABA-protein interaction at all concentration levels; but with RSA, low concentrations ($\leq 1 \times 10^{-3}$ M) of CPMPA may actually have enhanced the HBABA-protein interaction. She rationalized this unusual observation on the basis that CPMPA might be able to cause a small molecular perturbation in the RSA molecule which liberates additional sites on the protein for HBABA binding. Since all her protein binding data are calculated from spectrophotometric measurements which is indirect in nature and may reflect not only changes in number of molecules bound but also changes in the nature of the binding, we looked at this phenomenon using a direct measure of the competitive binding of CPMPA and HBABA to RSA at the same low concentration range of CPMPA ($\leq 1 \times 10^{-3}$ M).

For this purpose, the "Consecutive Steady-state Technique" was used; concentrations of HBABA and of RSA are twice those in Hermann studies (116) for sensitivity of measurement but the ratio of concentration of HBABA to RSA is kept the same, i.e., about 1.067; the concentration of CPMPA was increased from
zero to $23.8 \times 10^{-4}$ M which falls into the low concentration range specified by Hermann (116). The results obtained are given in Figure 40. The same general observation was obtained for potassium ion and sodium ion buffers. Very reproducible data were observed in repeat experiments.

Apparently, two linear relationships are followed between the binding ratio of HBABA to RSA and the ratio of CPMPA to HBABA. The binding of HBABA to RSA is decreased by CPMPA; the greater the concentration of CPMPA, the less the HBABA bound. This linear displacement of HBABA by CPMPA from RSA was also observed in Hermann's study with BSA in the same concentration range when same type of plot is made. The slope of the line in the case of RSA obtained in the present system is about 2-fold that in the case of BSA obtained from Hermann's report.

In addition, the same type of competition experiments was also carried out in the same concentration range by Nazareth (117) in this laboratory with a "Diafiltration Technique". One set of his data (which is also reproducible) is presented also in Figure 40. Perfect agreement between these two sets of data, when a same cationic species was used, is observed.
Figure 40  Competitive binding of CPMPA and HBABA to RSA as a function of cationic species: ○ Na⁺ ion studied by Diafiltration technique (Reference 117) is also included for comparison.

(CPMPA) : 0 - 2.38 x 10⁻³ M
(HBABA) : 1 x 10⁻⁴ M
(RSA) : 9.38 x 10⁻⁵ M
The use of different cationic species result in a different binding ratio between HBABA and RSA as in the case of Nitrophenol-HSA and HBABA-BSA systems discussed earlier, but does not affect the pattern of competitive binding between CPMPA and HBABA to RSA. The same slope is obtained for both K+ and Na+ ions (Figure 40).

What is most significant in these results is that we have direct proof that CPMPA displaces HBABA from RSA even at low concentrations of CPMPA. It may therefore be possible that what Hermann observed for RSA spectrophotometrically was a change in binding site nature and definitely not in the number of binding sites.

Two techniques used in the present investigation come to the same conclusion, that is, that the CPMPA competes with HBABA for the same binding sites on protein molecules. The higher the concentration of CPMPA, the lower the number of HBABA molecules bound. This same competitive inhibition by CPMPA occurs in both Rat and Bovine Serum Albumins.

Many drugs are bound to serum albumin (115) and some are believed to act by replacing the therapeutically active substance on the plasma protein. During the study of competitive binding of CPMPA and HBABA to BSA and RSA it was observed that release of the
"active substance", HBABA, occurs immediately and increases in direct proportion to the increase in concentration of the "inhibitory substance", CPMPA. This behavior is the same as that reported by Hermann (116) for BSA. This study points out that two conclusions may be made: (i) "Consecutive Steady-state Technique" plus "Rotating-disc Electrode Polarography" is a practical methodology for studying the drug-protein interaction and (ii) Competitive binding phenomena may be utilized to examine the binding character of a non-electroactive drug species.

The technology applied in the present studies allows a direct detection and measurement of free HBABA concentration, thus more reliable results would be expected as compared to the indirect spectrophotometric methods used by Hermann (113, 116). In addition, the binding character reported in the present studies are calculated through the use of a computer programmed according to Eq. (57) for 2 classes of sites. This is quite different from data extrapolated from graphical Langmuir plots used by Hermann where only low r value data were used. The present data analysis and interpretation is far more precise and accurate.
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