Exchange Diffusion of Dopamine Induced in Planar Lipid Bilayer Membranes by the Ionophore X537A

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ABSTRACT The ionophore X537A causes a large increase in the [14C]dopamine (a catecholamine) permeability of planar bilayer membranes. Dopamine transport increases linearly with the ionophore concentration. At relatively high concentrations in the presence of dopamine, the ionophore induces a conductance which is nearly ideally selective for the dopamine cation. However, the total dopamine flux as determined in tracer experiments is not affected by an electric field and is over 10^4 times larger than predicted from the estimated dopamine conductance. Increasing the dopamine concentration on the side containing radioactive dopamine (the cis side) saturates the dopamine transport. This saturation is relieved by trans addition of nonradioactive dopamine, tyramine, H^+, or K^+. With unequal concentrations of dopamine cis and trans (49 and 12.5 mM), the unidirectional dopamine fluxes are equal. Increasing H^+ cis and trans decreases dopamine transport. It is concluded that at physiological pH, the X537A-induced transport of dopamine occurs via an electrically silent exchange diffusion of dopamine cation with another cation (e.g., dopamine^+, H^+, or K^+). X537A induces a Ca^{2+}-independent release of catecholamines from sympathetic nerves by interfering with intracellular storage within storage vesicles (R. W. Holz. 1975. Biochim. Biophys. Acta. 375:138-152). It is suggested that X537A causes an exchange of intravesicular catecholamine with a cytoplasmic cation (perhaps K^+ or H^+) across the storage vesicle membrane.

INTRODUCTION

The carboxyl-containing ionophore X537A complexes with and transports Ca^{2+} and other divalent ions across cell membranes. It has been widely used in the study of Ca^{2+}-dependent phenomena in biological systems and has proved particularly useful in studies concerning the Ca^{2+}-dependent exocytotic release of hormones and neurotransmitters from cells and nerve terminals (e.g., Casswell and Pressman, 1972; Pressman, 1973; Estrada et al., 1974; Cochrane and Douglas, 1974). However, one of the unexpected findings has been that X537A releases catecholamine neurotransmitters from both peripheral and central sympathetic nerve terminals in a calcium-independent manner (Schaffer et al., 1974; Thoa et al., 1974; Holz, 1975). It has been recognized that X537A also complexes univalent ions including amines and catecholamines. It appears from studies in synaptosomes and isolated storage vesicles that X537A increases the
permeability of intracellular and intraterminal storage vesicles to catecholamines (Johnson and Scarpa, 1974; Holz, 1975).

In order to understand the biological actions of X537A, a number of studies have been undertaken on planar bilayer membranes (Celis et al., 1974; Schadt and Haeusler, 1974; Kafka and Holz, 1976); most of these studies were concerned with the electrical effects of the ionophore. However, it was apparent from the study of Kafka and Holz (1976) that at physiological pH most of the effect of X537A on membrane permeability is not measurable by electrical means. Furthermore, the evidence suggested that the dopamine transport induced by X537A occurred via an exchange diffusion mechanism. In the present study these results are extended with special attention paid to a comparison of the dopamine-dependent electrical conductance with the tracer dopamine permeability induced in bilayer membranes by X537A. Exchange diffusion is explicitly demonstrated as are trans effects of transportable cations on dopamine transport.

**Model for Tracer Dopamine Transport Induced by X537A**

Kafka and Holz (1976) using radioactive dopamine, demonstrated that dopamine flux increased proportionately with the X537A concentration in the aqueous solutions, which suggested that dopamine was being transported as a 1:1 complex with X537A. An electric field across the membrane had no effect on the dopamine transport; thus, in a transport cycle, no significant net charge was transferred across the membrane. The addition of nonradioactive dopamine greatly enhanced the transport from the cis to the trans side. This trans effect of a transportable cation suggests that returning the carrier to the cis side could become rate limiting in a transport cycle. At pH 7.2, at which these experiments were done, X537A (pK = 3.7; Degani and Friedman, 1974) is mainly negatively charged and dopamine mainly positively charged (catechol pK = 8.9, amine pK = 10.6) (Fig. 1A). Thus, a neutral complex of one negatively charged X537A and one positively charged dopamine could be formed. This form (without net charge) should be more soluble than a charged complex in the hydrocarbon interior of the membrane and is therefore more likely to be the main transporting species. A transport scheme with this neutral complex is shown in Fig. 1B.

The neutral X537A-dopamine complex could be formed either in aqueous solution immediately adjacent to the membrane and then partition into the membrane or by an interfacial reaction of X537A in the membrane phase and dopamine in the aqueous phase. Once in the membrane the complex diffuses across to the trans side and dissociates into dopamine cation and X537A anion, either at the interface or after partitioning into the aqueous solution. Because an electric field has no effect on the transport, it is unlikely that the X537A anion can return directly to the cis side. Instead, it must complex with a transportable cation to form a neutral complex, the complex then returns to the cis side and dissociates, releasing the cation and reforming the X537A anion. The cycle is completed. The accelerative effect of adding nonradioactive dopamine trans

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1 In a tracer experiment cis is defined as the side to which the radioactive isotope is added. trans is the opposite side. See Materials and Methods.
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(Kafka and Holz, 1976) is readily explained. The trans dopamine is counter transported cis and in the process enhances the rate of return of the carrier to the cis side.

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**FIGURE 1.** A, Chemical formulae of X537A (Johnson et al., 1970) and dopamine. B, Model for dopamine transport by X537A. XH = X537A, X = X537A with carboxyl group dissociated, D = dopamine cation, H = hydrogen ion. Subscript a refers to aqueous solution. Rate constants are noted above arrows. The possible complexation in aqueous solution of X537A and dopamine is not shown.

In the absence of another transportable cation trans, H⁺ is a likely ion to be countertransported, since it readily associates with the carboxyl group of X537A. The effect of trans addition of H⁺ ions and other ions is investigated in this paper. Finally, increasing the [H⁺] cis and trans should inhibit dopamine transport by X537A by competing with dopamine for the ionophore. This also was examined.
Although many of the results reported in this paper are qualitatively in agreement with this relatively simple exchange diffusion model, there are important discrepancies. However, the model is a useful focal point and is considered in more detail in the Discussion.

**MATERIALS AND METHODS**

Experiments were performed at room temperature in a chamber with two compartments each containing 2 ml of buffered solution. The compartments were separated by a Teflon partition 100 or 125 µm in thickness with a 1.37 mm diam hole in the center. Each compartment was stirred with small magnetic stirrers at 250 rpm. Membranes were formed by the brush technique of Mueller et al. (1963) from 2.5% bacterial phosphatidyl ethanolamine (PE) (Supelco, Inc., Bellefonte, Pa.) in decane or from a mixture of egg lecithin (Supelco), and cholesterol (twice recrystallized from ethanol) in decane (2.5% lecithin, 1.25% cholesterol) (LC). The membrane areas were 0.008-0.009 cm². Two calomel electrodes, which made contact with the solution via agar bridges, were connected to a combination voltage clamp-current clamp device to measure the electrical properties of the membrane (Montal and Mueller, 1972). The diffusion potential across the membrane was measured in the current clamp mode at zero current with a 101° resistor or an infinite resistance (open circuit) as the fixed resistor. The conductance of the unmodified membrane was 2-5 × 10⁻¹⁸ Ω⁻¹.

Tracer experiments were performed as described by Holz and Finkelstein (1970). Generally, a membrane was formed and X537A was added in equal concentrations to the cis and trans aqueous compartments. After 15-20 min, 10-20 µCi of isotope were added cis to begin the tracer experiment (zero time). In some experiments isotope was added before X537A within 5 min of membrane thinning. After 30 s a 0.05-ml sample was taken from the cis side. Thereafter 0.05-ml samples were taken periodically from the trans side and replaced with an equal volume of identical buffer to prevent bulging of the membrane. The radioactivity of the samples was determined in 9 ml Aquasol (New England Nuclear, Boston, Mass.) in a scintillation counter. The flux of dopamine was determined from the rate of appearance of isotope in the trans compartment. The dilution caused by replacement of trans solution was corrected for in calculations of the flux. Unstirred layer corrections were routinely made on the [14C]dopamine fluxes (Holz and Finkelstein, 1970) and were generally less than 15%. It was assumed that the unstirred layer was 150 µm and the diffusion coefficient of dopamine was 0.7 × 10⁻⁵ cm²/s. When dopamine concentrations cis were increased during the course of an experiment, the absolute fluxes were calculated by using the modified specific activity of the [14C]dopamine.

Solutions contained 0.1-0.2 mm ascorbic acid (prepared daily) to prevent oxidation of the catecholamine. The buffers used in these experiments, triethanolamine-Cl and tetramethylammonium-PIPES (piperazine-N-N'-bis[2-ethanesulfonic acid]), contain bulky cationic groups which should not complex appreciably with X537A. Concentrations used are specified in the figures. Aside from H⁺, no other cations were present unless indicated.

(Ethylamine-1-[¹⁴C]) or (ethylamine-2-[¹⁴C])-dopamine hydrochloride (50-59 mCi/mmol) (Amersham/Searle Corp., Arlington Heights, Ill.) was dissolved in 0.01 M HCl containing 10% ethanol. X537A was added from a concentrated solution in ethanol. X537A was a gift from Dr. Julius Berger, Hoffman-La Roche, Nutley, N. J.

**RESULTS**

**Electrical Effects**

In the absence of dopamine, addition of 5-10 µM X537A raises the conductance of PE or LC bilayers from 2-5 × 10⁻¹⁸ Ω⁻¹ to 0.7-7.0 × 10⁻⁹ Ω⁻¹. If dopamine is
then added, the conductance increases linearly with the dopamine concentration (Fig. 2 A). This conductance requires both X537A and dopamine, since in the absence of X537A dopamine causes little or no conductance increase. In the presence of dopamine, conductance increases proportionally with the ionophore concentration raised to the second to third power (mean ± standard deviation for three experiments is 2.5 ± 0.4) (Fig. 2 B). When the dopamine concentration was greater in the cis compartment, a potential appeared across the membrane.

The sign (cis side negative with respect to the trans side) and the magnitude of the potential (55 mV/10-fold concentration gradient) indicated that the membrane was almost ideally selective to the dopamine cation (Fig. 2 C). Hence the conductance of the bilayer in the presence of 5–10 μM X537A and 10–40 mM dopamine is a dopamine conductance. In the tracer dopamine experiments to be described, the X537A concentrations were a small fraction of those used to investigate the electrical effects of the ionophore. Although there were small conductance changes upon addition of these lower concentrations of X537A, they did not increase in response to dopamine addition and were, therefore, probably caused by the ionophore alone. From the data in Fig. 2, one extrapolates that at 0.5–1 μM X537A in the presence of 20 mM dopamine, the dopamine conductance should be approximately equal to the unmodified membrane conductance.
Time Course of $[^{14}C]$Dopamine Flux

In the absence of ionophore, $[^{14}C]$dopamine flux was not measurable ($P_d < 2 \times 10^{-6} \text{ cm/s}$). Upon addition of 1 $\mu$M X537A cis and trans\textsuperscript{2} the dopamine flux immediately increased but did not become constant until 10–20 min, after which time it remained unchanged for over 1 h (Fig. 3). The lag in attaining a constant dopamine flux was not seen in a previous study (Kafka and Holz, 1976). The lag was not due to a difference in lipids, since it was seen with lecithin-cholesterol bilayers which were also used in the previous study. Membrane area was un-

![Figure 3](image-url)

**Figure 3.** Time course of effect of X537A on $[^{14}C]$dopamine transport. A phosphatidyl ethanolamine membrane was formed in the presence of 100 mM triethanolamine-Cl, 1 mM dopamine (cis and trans), and 0.2 mM ascorbic acid, pH 7.2. $[^{14}C]$Dopamine was added cis (zero time). There were 581,000 cpm/0.05 ml cis. At 1 min X537A was added to the aqueous solution. Membrane area was 0.008 cm\(^2\). • radioactivity trans; △ membrane conductance.

changed during the course of the experiments. There appears to be a time-
dependent change in the bilayer since, once a steady flux of dopamine is reached, further additions of X537A result in an immediate (within 1 min) increase in flux which is seemingly constant (Fig. 4). In most of the flux experiments the $[^{14}C]$dopamine was added 15–20 min after the membrane had thinned and after X537A had been added in order to avoid the lag phase. With these low ionophore concentrations the conductance bears little relationship to the dopamine flux. For example, in Fig. 3 the conductance continues to rise after the dopamine flux (proportional to the rate of appearance of the radioactivity into the trans compartment) reaches a constant value.

\textsuperscript{2} Dopamine flux also increases when X537A is added to the trans side only. The dopamine flux was approximately half the flux compared with the ionophore present cis and trans. The ionophore must cross to the cis side and be at a significant concentration cis to transport measurably $[^{14}C]$dopamine from the cis to the trans side. Thus, X537A must be more permanent through the membrane than through the unstirred layer.
Effects of Adding Nonradioactive Dopamine

Kafka and Holz (1976) demonstrated that the dopamine flux increases less than proportionally as the cis dopamine concentration is raised and that when the trans dopamine concentration was increased, a many-fold increase in dopamine flux resulted. Since these effects are important evidence for the existence of exchange diffusion, they were further examined in the present study (Fig. 5). On a single membrane the dopamine concentration was increased cis with nonradioactive dopamine. The [$^{14}$C]dopamine flux decreased as the dopamine concentration cis increased (Fig. 5 A). However, when the change in specific activity of the dopamine is taken into account, one finds that the total dopamine flux increased with the dopamine concentration but in a less than proportional manner (Fig. 5 B). The points fit a Michaelis-Menten saturation curve as shown in the Woolf plot (Fig. 5 C). According to the exchange diffusion model the saturation is occurring because the carrier, by transporting the dopamine from the cis to the trans side, is actually being depleted from the cis side of the membrane. If the transport is being limited by the return of the carrier from the trans to the cis side, addition of a transportable cation trans should enhance the transport.
Indeed, as has been previously reported, the addition of dopamine \textit{trans} increased the transport more than threefold (Fig. 5 A). The new flux is actually off scale in Fig. 5 B.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Effects of increasing the dopamine concentration \textit{cis} and \textit{trans} and of electric field on dopamine transport. A phosphatidyl ethanolamine membrane was formed in the presence of 100 mM triethanolamine-Cl and 0.2 mM ascorbic acid, pH 7.2. X537A, 0.5 \textmu M, was added \textit{cis} and \textit{trans}. After 10 min dopamine was added \textit{cis} to make the concentration 0.5 mM. After 15 min \textsuperscript{14}C)dopamine (56 mCi:mmol) was added \textit{cis} (zero time, Fig. 5 A) to make the total dopamine concentration 0.57 mM. There were 405,000 cpm/0.05 ml \textit{cis}. Dopamine fluxes were determined with various dopamine concentrations as indicated in the figure. From 0 to 48.5 min the membrane potential was 0 mV. At 48.5 min the membrane potential was clamped continuously at 50 mV (cis+). Membrane area was 0.008 cm\textsuperscript{2}. Membrane conductance from 0 time to the end of the experiment was 2.4 \times 10^{-9} \Omega^{-1}. A, Radioactivity \textit{trans} vs. time. B, Total dopamine flux vs. total dopamine concentration \textit{cis}. C, Woolf kinetic plot.}
\end{figure}

This experiment also confirms that the dopamine flux is not affected by an electric field. 50 mV had no effect on the dopamine flux with 15.5 mM dopamine \textit{cis} and \textit{trans} (Fig. 5 A). If the dopamine-X537A complex had a positive charge and followed the Nernst-Planck flux equation, then a 50-mV potential would have resulted in a 2.3-fold increase in the dopamine flux.\textsuperscript{3}

\textsuperscript{3} When ion flow is coupled as in exchange diffusion, the transport is not described by the Nernst-Planck flux equation. If none of the transported species in a transport cycle is charged, then an electric field should have no effect.
Saturation also occurred when nonradioactive dopamine was added both cis and trans (Fig. 6) but it occurred at much higher dopamine concentrations. Table I summarizes the saturation data. Both the $K_m$ (concentration of dopamine giving one-half maximal dopamine transport) and the $J_{\text{max}}$ (maximal flux) determined with dopamine cis and trans are 10-fold greater than the $K_m$ and $J_{\text{max}}$ determined with dopamine cis only. To determine whether the saturation with dopamine cis and trans occurs because of a limited solubility of the X537A-
dopamine complex in the membrane, the effect of increasing the X537A concentration on dopamine flux was investigated in the presence of a saturating concentration of dopamine (71 mM) cis and trans (Fig. 4). The transport is linear with X537A concentration; thus, the saturation does not occur because of a limited solubility of the complex in the membrane. It is also possible that lack of equilibrium between the ionophore in bulk aqueous solution and the lipid-decane torus around the bilayer (acting as a “sink” for the ionophore) (Hladky, 1973) could lead to a limitation of the amount of ionophore that could enter the bilayer; saturation could result as the dopamine concentration is raised cis and trans. This was investigated by adding the ionophore to the membrane-forming solution and measuring the dopamine flux. With 1 mM X537A in the membrane-forming solution1 saturation also occurred at approximately 30 mM as the dopamine concentration cis and trans was increased. Thus the saturation is probably not due to torus effects. Another explanation may be that saturation occurs because of a saturation of X537A-dopamine complex formation in aqueous solution. Complexation of X537A with cations in aqueous solution has been studied by monitoring changes in the fluorescence of X537A accompanying complexation. Because dopamine absorbs strongly where X537A absorbs, it was not possible to study the effects of dopamine on the fluorescence of X537A.

**Demonstration of Exchange Diffusion**

The lack of linearity of dopamine transport with cis dopamine concentrations of millimolar or greater (Fig. 5, Table I) and the relief of this nonlinearity upon trans addition of dopamine suggest that with more than 10 mM dopamine cis and trans, even if the dopamine concentrations are unequal on both sides of the

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**TABLE I**  

| Dopamine cis | Dopamine cis and trans | Dopamine cis and trans |
|--------------|------------------------|------------------------|
| Kₘ (nM)  | J_max (mol/cm²s) | Kₘ (nM) | J_max (mol/cm²s) |
| 5.18 | 3.10 | 28.0 | 18.3 |
| 2.78* | 1.90* | 38.5 | 34.5 |
| 4.82 | 4.40 | 21.4* | 12.8* |
| 2.62 | 1.40 | 25.7* | 20.5* |
| 3.27 | 2.15 | 41.1* | 36.6* |

Mean±SEM 3.75±0.53 2.59±0.53 30.9±3.79‡ 24.5±4.68§

Phosphatidyl ethanolamine membranes were formed in the presence of 100 mM triethanolamine-Cl and 0.2 mM ascorbic acid, pH 7.2. The X537 A concentration was 0.5 μM cis and trans. Kₘ and J_max were determined on single membranes from the unidirectional dopamine fluxes determined at different dopamine concentrations cis or cis and trans (see Figs. 5 and 6). Unless indicated by an asterisk, the values were obtained from fluxes at three different dopamine concentrations.  
* Fluxes determined at two concentrations of dopamine.  
‡ P<0.001 compared to the values with dopamine cis only.

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1 mM X537A in the membrane-forming solution gave dopamine fluxes approximately equal to those obtained with 0.5 μM X537A in the aqueous solutions.
membrane, dopamine cis should exchange quantitatively with dopamine trans.\(^5\)

To demonstrate equal unidirectional fluxes of dopamine in the two directions across the membrane with different cis and trans dopamine concentrations, the flux from cis to trans was investigated on several membranes with a high concentration of dopamine cis (49 mM) and a low concentration trans (12.3 mM). On other membranes the flux from cis to trans was investigated with a low concentration of dopamine cis (12.3 mM) and a high concentration trans (48.3 mM) (Table II). The fluxes are equal. Thus, exchange diffusion of dopamine for dopamine does occur under these conditions. A similar experiment was done with 49 mM dopamine on one side and 0.1 mM dopamine on the other (a concentration at which saturation is not significant). Two membranes were

| Table II | Dopamine Fluxes with Unequal and Equal Concentrations of Dopamine cis and trans |
|----------|--------------------------------------------------------------------------------|
|          | DA\(_{cin}\) \# DA\(_{trans}\) | DA\(_{cin}\) = DA\(_{trans}\) |
|          | cis | trans | \(J_{DA}\) \(mol\ (s\cdot cm^{-2})^{-1} \times 10^{13}\) | \(J_{DA}\) \(mol\ (s\cdot cm^{-2})^{-1} \times 10^{13}\) |
| No. membranes |    |      |              | 0.1 mM DA | 12.5 mM DA | 50 mM DA |
| mM        |    |      |              |          |            |          |
| 4         | 49.2 | 12.3 | 7.99 ± 0.50  | –        | 5.21 ± 0.48 | –        |
| 3         | 12.3 | 48.3 | 7.72 ± 0.55  | –        | 4.82 ± 0.43 | –        |
| 1         | 49   | 0.1  | 4.44         | 0.0886   | –          | 19.9     |
| 1         | 0.1  | 49   | 0.228        | 0.0895   | –          | 20.9     |

Phosphatidyl ethanolamine membranes were formed in the presence of 100 mM triethanolamine-CI, and 0.2 mM ascorbic acid. \(^{[14C]}\)Dopamine was added after a 20-min incubation with 0.5 \(\mu\)M X537A cis and trans. Dopamine fluxes were determined with various concentrations of dopamine cis and trans. See text for explanation.

compared. Fluxes were first measured with 0.1 mM dopamine cis and trans, then with 49 mM either cis or trans and 0.1 mM trans or cis, and finally with 49 mM cis and trans. With 0.1 mM dopamine cis and trans the membranes had equal fluxes, and with 49 mM dopamine cis and trans the membranes had equal fluxes. However, the unidirectional cis to trans flux with 49 mM dopamine cis and 0.1 mM dopamine trans was 20-fold greater than with 0.1 mM cis and 49 mM trans. In this situation dopamine is not significantly exchanging with dopamine. A similar situation was encountered when dopamine was present only in the cis solution (Fig. 5); dopamine crossed the membrane although there was no dopamine trans to exchange with. If exchange diffusion is occurring under these conditions, dopamine must be exchanging with another ion. One possible cation is the charged form of the buffer triethanolamine. When the buffer concentration was doubled from 50 mM to 100 mM trans (the concentration cis remained 50 mM) there was no change in the dopamine flux with 10 mM dopamine cis (Table III). It is unlikely that triethanolamine significantly exchanges with dopamine. Another possible cation is \(H^+\), especially since it can associate with the carboxyl group of the negatively charged ionophore.

\(^5\) With identical solutions cis and trans dopamine must move at equal rates in both directions regardless of the concentration.
Effect of Changing $H^+$ trans

If $H^+$ can exchange with dopamine across the membrane, then in the absence of dopamine trans, if $H^+$ is increased trans, the cis to trans transport of dopamine should increase. Conversely, if the $H^+$ is decreased trans, the dopamine flux should decrease. Indeed, when the $H^+$ concentration trans was increased eightfold (pH change from 7.2 to 6.3) the dopamine flux increased fourfold (Fig. 7 A). The effects on the dopamine flux of changing $H^+$ trans are summarized in Fig. 7 B. Decreasing the $H^+$ concentration trans decreases the flux and increasing the $H^+$ concentration trans increases the flux. One might argue that the ability of $H^+$ trans to enhance the flux of dopamine is a nonspecific effect on membrane properties. This is unlikely for the following reasons. (a) When $H^+$ cis was increased threefold the dopamine flux decreased by 25% instead of increasing. This indicates a dependence of the effect on the side to which the $H^+$ is added. (b) It is possible that changing $H^+$ changes the surface charge and thereby changes the surface potential of the PE membranes. This was investigated by measuring changes in the valinomycin-$K^+$ or nonactin-$K^+$ conductance caused by $H^+$ changes (McLaughlin et al., 1970). The buffers used were identical to those used in the flux experiments except for the addition of 2.5–10 mM KCl and 0.1 $\mu$M valinomycin or 5 $\mu$M nonactin. $H^+$ changes over the range investigated in the flux experiments resulted in less than a 15% change in the $K^+$ conductance. Thus, it is unlikely that $H^+$ effects on dopamine flux are related to changes in surface potential. (c) With high concentrations of dopamine cis and trans where dopamine-dopamine exchange should be much greater than dopamine-$H^+$ exchange, $H^+$ concentration changes should have no effect. Indeed, in the presence of 72 mM dopamine cis and trans, increasing $H^+$ trans 2.5-fold had no effect on the dopamine flux. Therefore, the enhancement of dopamine flux by trans addition of $H^+$ is a specific effect on dopamine transport by X537A and is

### Table III

**EFFECTS OF TRANS ADDITIONS OF VARIOUS CATIONS ON DOPAMINE FLUX CIS TO TRANS**

| Changes in dopamine flux | 0-10% increase | 10-100% increase | >100% increase |
|--------------------------|----------------|-----------------|---------------|
| Triethanolamine* *       | K* †          | Dopamine* †     |
| Na** *                  | H** †         |                |
| Ca** †                  | Tyramine* *   |
| Ba** †                  |                |

Phosphatidyl ethanolamine membranes were equilibrated with 0.5-1.0 $\mu$M X537A cis and trans for 15-20 min. Unidirectional dopamine flux was initially measured with dopamine cis only. The test cation was then added trans to give a concentration of 25 mM or greater. The effects of triethanolamine were tested by first measuring the dopamine flux with 50 mM triethanolamine-Cl and 0.2 mM ascorbic acid, pH 7.2, cis and trans. Then concentrated triethanolamine-Cl was added trans to give a concentration of triethanolamine-Cl trans of 100 mM (pH 7.2). The other experiments were all done in 100 mM triethanolamine-Cl, and 0.2 mM ascorbic acid, pH 7.2, cis and trans.

* [DA] cis was 10–12.5 $\mu$M.
† [DA] cis was 0.07–0.1 $\mu$M.
Figure 7. The effect of changing H$^+$ trans. A. Enhancement of dopamine transport by increasing the H$^+$ concentration trans. A phosphatidyl ethanolamine membrane was formed in 50 mM tetramethylammonium-PIPES and 0.2 mM ascorbic acid, pH 7.23. X537A, 0.5 μM, was added cis and trans. After 12 min dopamine was added cis to make the concentration 12.5 mM cis. After 17 min (zero time) $^{14}$C]dopamine was added cis. There were 547,000 cpm/0.05 ml cis. At 12 min HCl was added trans to reduce the pH to 6.31 (H$^+$ concentration increased 8.4-fold). The membrane conductance was $1.5 \times 10^{-9}$ Ω$^{-1}$ throughout the experiment. The membrane area was 0.008 cm$^2$. Increasing the H$^+$ concentration trans increased the dopamine flux from $1.59 \times 10^{-10}$ mol (s·cm$^{-2}$)$^{-1}$ to $6.29 \times 10^{-10}$ mol (s·cm$^{-2}$)$^{-1}$. B. Relative dopamine flux vs. H$^+$ concentration trans. Fluxes were determined as in Fig. 7A. Phosphatidyl ethanolamine membranes were formed at pH 7.2 ([H$^+$] = 6.31 × 10$^{-8}$ M). X537A, 0.5 μM, was present cis and trans. Dopamine was added to make the cis concentration 12.5 mM. After a 20-min preincubation in X537A, [H$^+$]trans was 6.31 × 10$^{-8}$ M. Each point is the average of two experiments with the vertical bars indicating the range. Experiments in which the H$^+$ concentration was increased trans were performed in 50 mM tetramethylammonium-PIPES and 0.2 mM ascorbic acid. The H$^+$ concentration was increased with HCl. Experiments in which the H$^+$ concentration was decreased were performed in 100 mM triethanolamine-CI and 0.2 mM ascorbic acid. The H$^+$ concentration was reduced with triethanolamine base.
strong evidence for $\text{H}^+$-dopamine exchange in the absence of another exchangeable cation trans.

**The Ability of Other Ions trans to Enhance Dopamine Flux**

The ability of a number of ions on the trans side of the membrane to enhance the dopamine flux was investigated (Table III). Only tyramine was able to give comparable effects to dopamine and $\text{H}^+$. $\text{K}^+$ gave moderate effects. The other ions had no appreciable effects. The absence of effects of $\text{Ca}^{++}$ which can be transported by X537A will be considered in the Discussion.

**Effect of Changing $\text{H}^+$ on cis and trans Sides**

If the major transporting species is dopamine complexed with an X537A anion, then as the $\text{H}^+$ concentration is raised cis and trans (pH lowered) $\text{H}^+$ should more effectively compete with dopamine for the ionophore, and the dopamine flux should fall. Indeed, dopamine flux falls as the $\text{H}^+$ concentration increases (Fig. 8A). It is not possible to define a pH at which the dopamine flux is half-maximal, since the flux does not level off at low $\text{H}^+$ concentrations (high pH).

**DISCUSSION**

Although X537A has been used widely in biological experiments on the assumption that it acts as a $\text{Ca}^{++}$ ionophore, there are clear indications that it can have other effects. In some experiments X537A seemed to act as a catecholamine ionophore to increase the catecholamine permeability of biological membranes (Johnson and Scarpa, 1974; Holz, 1975). Subsequent bilayer studies demonstrated that the ionophore can increase the permeability of bilayer membranes to catecholamine (Schadt and Haeusler, 1974; Kafka and Holz, 1976). This study extends these results and shows that the tracer dopamine flux induced by X537A occurs via an electrically silent exchange diffusion of dopamine cation cis with another transportable cation trans. This conclusion is based upon: (a) the lack of effect of an electric field on the tracer dopamine-transport; (b) the observed tracer dopamine flux being many orders of magnitude larger than the flux estimated from the electrically measurable dopamine transport (see below); (c) the demonstration of equal unidirectional fluxes of dopamine across the membrane with different dopamine concentrations cis and trans; and (d) the trans effect of various transportable cations of enhancing many-fold the dopamine flux. These various points will be considered in more detail below.

**Evidence for Carrier-Mediated Transport**

The effect of X537A to increase the tracer dopamine permeability could result from the ionophore causing channel formation or from the ionophore acting as carrier for dopamine. The linearity of the tracer flux with the X537A concentration and the small diameter of the ionophore (12 Å from a CPK model of the head to tail hydrogen-bonded ionophore molecule) compared to the thickness of the bilayer (approximately 50 Å) suggests that the ionophore acts as a mobile carrier. It seems unlikely that the ionophore itself could span the membrane to form a channel. Furthermore, if channel formation were to occur and to account for both the tracer flux and the dopamine conductance, unless there were different type channels responsible for each, they both should increase

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with the same X537A stoichiometry. In fact, the dopamine conductance increases with the X537A concentration raised to the second to third power, whereas the tracer flux increases linearly with the X537A concentration.  

The Relationship of the Dopamine Conductance and the Dopamine Tracer Flux  
Although dopamine is mainly positively charged at pH 7.2 at which most of the experiments were done, dopamine tracer flux was not affected by an electric field. This electrically “silent” transport can be compared to the electrically measurable dopamine transport, as reflected by the dopamine conductance, using the relation (Hodgkin and Keynes, 1955):  

$$g_{DA} = \frac{Z^2F^3}{kT} J'_{DA}$$ (with equal dopamine concentrations cis and trans),

*For example, the channel-forming polyene antibiotics nystatin and amphotericin B increase the electrical conductance and the tracer ion, nonelectrolyte, and water permeabilities of planar bilayer membranes. All increase with the same high power (4th-12th depending upon the lipid) of the antibiotic concentration (Holz and Finkelstein, 1970).
where $g_{DA}$ is the small signal dopamine conductance; $Z$, the charge; $F$, the Faraday constant; $R$, the gas constant; $T$, the temperature (°K); and $J'_{DA}$ the predicted dopamine flux. At pH 7.0-7.2 through either LC or PE membranes, the actual dopamine flux is at least $10^6$ times larger than predicted from the dopamine conductance (see Appendix). The enormous disparity between the actual flux and the flux predicted from the dopamine conductance strongly suggests that there is tight coupling of ion flow in opposite directions, i.e., exchange diffusion.  

Additional Evidence for Exchange Diffusion

The quantitative exchange of dopamine with dopamine across the membrane was explicitly demonstrated with unequal dopamine concentrations cis and trans. In the absence of dopamine trans, increases in the trans H$^+$ concentration resulted in large increases in the dopamine flux. Several other ions were also investigated for their ability, when added trans, to increase the dopamine flux by increasing the rate of return of the carrier. Tyramine (a primary amine which is positively charged at pH 7.2) and K$^+$ were able to significantly increase dopamine flux, thus suggesting that they are being countertransported.

Although X557A can transport Ca$^{++}$ across bilayers (Kafka and Holz, 1976), Ca$^{++}$ was not able to enhance the dopamine flux. Quantitatively, however, the results are consistent. If one extrapolates from the Ca$^{++}$ flux experiments of Kafka and Holz to the present experiments where lower ionophore and higher Ca$^{++}$ concentrations were used, one calculates that the Ca$^{++}$ flux would be less than the dopamine flux with either 0.1 mM or 10 mM dopamine cis. Hence, under the present conditions the Ca$^{++}$ flux was not large enough to enable Ca$^{++}$ to exchange significantly with dopamine.

Saturation with Increased Dopamine Concentrations

Two types of saturation were observed with dopamine. When dopamine was increased on the cis side only, saturation occurred that could be relieved by adding dopamine trans. This saturation was probably caused by the transport being limited by the return of carrier to the cis side. The return of the carrier was facilitated by its being able to return complexed with dopamine. Saturation also occurred when the dopamine concentrations were increased cis and trans. This saturation was not caused by dopamine altering the surface potential or fluidity of the membrane. Under the conditions in which tracer experiments were done, 75 mM dopamine cis and trans did not alter the valinomycin-K$^+$ conductance. It is also unlikely that saturation occurred because of torus effects (see Hladky, 1973), since saturation also occurred when the ionophore was added to the membrane-forming solution. Saturation could occur if, during the time course of an experiment (10-30 min), the ionophore and its complexes in the mem-

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One might argue that instead of exchange diffusion, diffusion of a neutral X557A-dopamine complex is occurring from the bulk aqueous solution cis to the bulk aqueous solution trans. Because of the limited rate of diffusion of such a complex across the aqueous unstirred layers, this mechanism could not account for the large dopamine fluxes observed. If one assumes a diffusion coefficient of $0.5 \times 10^{-5}$ cm$^2$/s for the dopamine-X557A complex, the maximal flux of the complex across an unstirred layer of 150 μm with 1 μM complex in the bulk solution would be $3 \times 10^{-10}$ mol (s⋅cm)$^{-2}$ (See Holz and Finkelstein, 1970). This is 0.05-0.0001 of the fluxes measured in the present experiments.
brane were not in equilibrium with the ionophore and its complexes in the aqueous solution immediately adjacent to the membrane. However, with identical solutions cis and trans, lack of interfacial equilibrium is unlikely since the rate constants for these membrane events are probably on the order of $10^{-4}$-$10^{-3}$S$^{-1}$ (Läuger, 1972)—many orders of magnitude faster than the time scale of the tracer experiments. It is possible that saturation of transport is a reflection of saturating X537A-dopamine complex formation in aqueous solution. Unfortunately, this could not be successfully investigated (see Results).

**Effect of Changing pH cis and trans**

Consistent with the model of H$^+$ competing with dopamine for the ionophore was the finding that increasing the cis and trans H$^+$ concentration caused a reduction in dopamine transport. This was found when the ionophore was added both to the membrane-forming solution and to the aqueous solutions. However, other interpretations are also possible. Changing H$^+$ will also change the concentrations of different species of dopamine$^8$ and this could affect transport. Fig. 8 B is a replot of the results from one membrane with the fluxes expressed relative to that at pH 7.0. Also plotted are the calculated results expected from the transport from three different classes of complexes in the membrane. One class is composed of neutral complexes (left curve) and another of complexes with one negative charge (middle curve), and the last of a complex with two negative charges (right curve). These plots are based on the assumption that the transport is proportional to the concentration of complex in the membrane and that the concentration of complex in the membrane is proportional to the product of the aqueous concentrations of a species of ionophore and a species of dopamine. If dopamine$^+$ complexes with X537A$^-$ as suggested in the model in Fig. 1 B, one would expect the data to fit the left curve in Fig. 8 B. The experimental data do not fit the plots for any of the types of possible complexes. It is possible that there is more than one transporting species. It is also possible that the concentration of complex in the membrane may not be proportional to the product of the concentration of a dopamine species and the concentration of an ionophore species in aqueous solution. However, X537A and dopamine can form neutral complexes in an organic phase (Holz, 1975), and it seems probable that a neutral complex would be the preferred species in the lipid membrane phase. Furthermore, since at pH 7 over 99.9% of the ionophore is negatively charged and over 98% of the dopamine is positively charged, it seems likely that the predominant neutral complex would be formed from these predominant forms. Hence, although a quantitative analysis at this point is incomplete, qualitatively the results are consistent with the transport being due, at least in part, to a neutral complex composed of an ionophore anion and a dopamine cation.

**Relationship to Previous Work**

Aside from Kafka and Holz (1976) there have been only two other studies

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$^8$ The catechol moiety of dopamine (Fig. 1 A) can be neutral or negatively charged (pK = 8.9), and the amine group can be neutral or positively charged (pK = 10.6) (Dawson et al., 1968). There are, therefore, four forms of dopamine; their relative concentrations are pH dependent.
investigating the action of X537A on bilayers. Celis et al. (1974) considered the electrical effects of X537A in the presence of various cations but not catecholamines. They did not investigate tracer permeability. Schadt and Haeusler (1974) examined the net transport of catecholamines across bilayers, but not the electrical effects of this transport nor the possibility of exchange diffusion. The results in both these studies are consistent with those herein reported.

**Biological Significance**

Exchange diffusion, which is responsible for the X537A-induced increase in dopamine permeability in bilayers, may also be important for the effect of

| TABLE IV |
|------------------|
| **COMPARISON OF X537A-INDUCED DOPAMINE TRANSPORT ACROSS BILAYERS WITH CATECHOLAMINE TRANSPORT ACROSS CATECHOLAMINERGIC STORAGE VESICLE MEMBRANES** |

| X537A-induced dopamine transport across bilayers | Transport across catecholamine storage vesicle membranes |
|-----------------------------------------------|-----------------------------------------------|
| Saturable (especially with cis addition)       | Saturable*‡                                   |
| Dopamine trans enhances transport             | Catecholamine outside enhances efflux§§        |
| Tyramine trans enhances transport             | Tyramine outside exchanges quantitatively with catecholamine§¶ |
| H+ trans enhances transport                   | H+ outside enhances release|| |
|                                               | ATP, Mg greatly enhances uptake of catecholamine from medium** |

* In presence of ATP, Mg.
‡ Phillips, 1974.
§ In absence of ATP, Mg.
¶ Taugner, 1972.
§§ Schumann and Philipp, 1962.
** Kirshner, 1962.

causing a Ca++-independent release of catecholamines from central and peripheral sympathetic nerves. X537A causes a release of catecholamine from storage vesicles within sympathetic nerve terminals and from adrenal medullary cells (Johnson and Scarpa, 1974; Schaffer et al., 1974; Holz, 1975). *In situ* X537A may induce H+ or K+ present within sympathetic nerve cells to exchange with catecholamine in storage vesicles, thus releasing catecholamine into the cytoplasm. Catecholamine could then exit from the cell via the intrinsic plasma membrane catecholamine transport system, via X537A-mediated transport, or because of nonspecific leakage.

It is interesting to compare the X537A-induced dopamine transport across bilayers with the intrinsic catecholamine transport system in catecholamine storage vesicle membranes (Table IV). Both are saturable processes which exhibit enhancement of unidirectional transport when tyramine or catecholamine is present trans. H+ has comparable effects in the two systems. Important differences, however, are that transport into storage vesicles is strongly stimulated by Mg++ and ATP and results in the concentration of catecholamine within the vesicle. A recent intriguing observation is the existence of a pH gradient across the chromaffin granule membrane which may be caused by an ATPase in

* Adrenergic storage vesicle in adrenal medullary chromaffin cells.
the membrane (Bashford et al., 1975; Johnson and Scarpa, 1976). It is possible that the "active" catecholamine inward transport may result from a coupling via exchange diffusion, with $\text{H}^+$ inside the granule being transported outward. Such a mechanism would be akin to the $\text{H}^+$-dopamine exchange induced by X537A in bilayers. This possibility is currently being investigated.

Exchange diffusion has been demonstrated in several biological membranes (Ussing, 1947; Garrahan and Glynn, 1967; Baker et al., 1969; Sjodin, 1971) and is undoubtedly an important biological transport mechanism. The present study is the first of its kind to investigate exchange diffusion induced by an ionophore across a planar bilayer membrane. Although it may be a useful model for biologically occurring exchange diffusion, there may be fundamental differences. The ionophore-induced exchange diffusion is carrier mediated. The anion exchange diffusion in the red blood cell, the best understood system, appears to occur because of a large protein (100,000 daltons) in the membrane (Rothstein et al., 1976). It seems unlikely that such a large structure, which itself can probably span the membrane, is acting as a mobile carrier. Instead, a channel is more appropriately envisioned. Nonetheless, it is noteworthy that the chloride exchange permeability in the red blood cell is $10^4$ times larger than the electrically manifest chloride permeability (Hunter, 1971). This large difference is comparable to the estimated $10^5$-fold difference between dopamine exchange and electrically manifest dopamine permeability demonstrated in these studies.

**APPENDIX**

**The Relationship Between the Radioisotopic Dopamine Flux and the Dopamine Conductance**

If one assumes that the dopamine conductance is described by the Nernst-Planck flux equations (Ussing, 1949), or by the independence principle\(^{10}\) (Hodgkin and Keynes, 1955), one can predict the unidirectional radioisotopic flux in the absence of an electric field from the small signal dopamine conductance by using the relation

$$ g_{\text{DA}} = \frac{Z_2F_2}{RT} J'_{\text{DA}} \quad \text{(with equal dopamine concentrations cis and trans)}, \quad (1) $$

where $g_{\text{DA}}$ is the small signal dopamine conductance; $Z$, the charge; $F$, the Faraday constant; $R$, the gas constant; $T$, the temperature ($\degree K$); and $J'_{\text{DA}}$ the predicted dopamine flux (see Hodgkin and Keynes, 1955). In tracer experiments, because of the low concentrations of X537A that were necessarily used, it was not possible to clearly identify a dopamine conductance. However, at higher ionophore concentrations a dopamine conductance was readily demonstrated that increased with the ionophore concentration raised to the second to third power. At the lower X537A concentration (1 $\mu$M) used in the tracer experiments, one extrapolates from Fig. 2 that the dopamine conductance was approximately $4 \times 10^{-16}$ $\Omega^{-1}$ or $4 \times 10^{-4}$ (\text{\Omega cm}$^2$)$^{-1}$ with 47 mM dopamine present. With lower dopamine concentrations it should be proportionately lower. Consider, for example, the experiments shown in Fig. 8 done in the presence of 1 $\mu$M X537A and 10 mM dopamine cis and trans. The dopamine conductance, $g_{\text{DA}}$, is estimated to be $1 \times 10^{-8}$ (\text{$\Omega$ cm}$^2$)$^{-1}$. Substituting this value of $g_{\text{DA}}$ into Eq. (1) and assuming that $Z = \pm 1$, the esti-

\(^{10}\) It is possible that the protein has attached to it a mobile moiety that acts similarly to a shuttling carrier within the structure of the protein.

\(^{11}\) Either formulation assumes that ions move independently of each other.
mated dopamine flux is calculated to be $3 \times 10^{-15}$ mol (s·cm$^{-2}$)$^{-1}$. The actual dopamine flux at pH 7.0 is $8 \times 10^{-10}$ mol (s·cm$^{-2}$)$^{-1}$ or $3 \times 10^5$ times larger than predicted. Calculations using data from PE membranes yield similar results.

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