A MODEL FOR THE DIFFUSION OF FLUORESCENT PROBES IN THE SEPTATE GIANT AXON OF EARTHWORM

Axoplastic Diffusion and Junctional Membrane Permeability

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ABSTRACT The diffusion of the three fluorescent probes dichlorofluorescein, carboxyfluorescein, and Lucifer Yellow within the septate median giant axon of the earthworm was monitored using fluorometric methods. A diffusion model was derived that allowed computation of the apparent axoplastic diffusion coefficient, junctional membrane permeability (septal membranes), and plasma membrane permeability for each probe. Dichlorofluorescein and carboxyfluorescein have similar apparent axoplastic diffusion coefficients, which were reduced by a factor of eight relative to that predicted from the Einstein-Stokes equation. Nonspecific reversible binding appears to be the major cause of the retarded diffusion coefficients. Junctional membrane permeability for dichlorofluorescein was 4.7 to 73-fold greater than that for carboxyfluorescein. This difference could not be explained on the basis of molecular size but can be explained by the difference in charge between the two molecules. Diffusion coefficients and junctional membrane permeabilities remained constant with time for both dyes. The diffusion of Lucifer Yellow within the axoplasm and permeability through the junctional membranes did not remain constant with time but declined. From this it was inferred that Lucifer Yellow experienced a slow, irreversible binding to axoplasmic elements. All three probes had finite plasma membrane permeabilities.

INTRODUCTION

The measurement of solute diffusion through intercellular pathways and cytoplasm is often complicated by cellular size and geometry. Cells are often small and the intercellular junctions occur randomly over the surface of cells. Within a tissue such as liver, diffusion of a solute can occur in three dimensions in vivo, or two dimensions in tissue culture. In either case the diffusion of a tracer molecule is determined as an effective or apparent diffusion coefficient. The determinants of the apparent diffusion coefficient are junctional membrane permeability, solute binding to cytosolic and membrane bound proteins, sequestration of the solute into intracellular compartments, tortuosity factors (cytoskeleton and intracellular organelles), and viscosity of the cytoplasm. If the junctional membranes are assumed to occur periodically and perpendicular to the long axis of a cylindrical tissue bundle (e.g., heart myocardial strips) then junctional permeabilities and apparent cytoplasmic diffusion coefficients can be estimated (Weingart, 1974).

Measurement of cytoplasmic diffusion coefficients of various probes in cells that are true syncytia (e.g., skeletal muscle cells) show a reduction in the diffusion coefficients by a factor of 2 to 100 times relative to those obtained in the aqueous phase (Kushmerick and Podolsky, 1969) or from the Einstein-Stokes equation. Since the diffusion coefficient of a solute molecule is inversely proportional to solvent viscosity (Einstein-Stokes equation, Robinson and Stokes, 1968) estimates of cytoplasmic viscosity are necessary in determining the other factors that influence the diffusion coefficient of a molecule within a cell. EPR studies in Myxicola axoplasm of two low molecular weight spin labels revealed a microviscosity of only 0.02 poise (Rubinson and Baker 1979). Dintenfuss (1968) measured the viscosity of erythrocyte cytoplasm at 37°C and found it to be four times greater than water. Cokelet and Meiselman (1969) obtained similar results. More recently, Mastro and Keith (1984) performed electron-spin resonance experiments on cultured cells to assess the rotational and translational motion of solutes within cells. Their studies demonstrate that the viscosity of cytoplasm is greater than the viscosity of water by a factor of 2 to 4 for both motions. Removal of solvent by hypertonic treatment further decreased translational motion by fourfold but suppressed rotational motion only slightly (20% decrease), leading the authors to suggest an increase in the density of cytoplasmic barriers. Greater reductions than those due to viscosity in apparent cytoplasmic diffusion coefficients suggest that some form of solute entrapment occurs within the cytosol, or that there exist cytoplasmic barriers (e.g., tortuosity) to translational motion of solutes.
If intercellular transfer of solutes is mediated by aqueous channels (Bennett, 1977; and Loewenstein, 1975 and 1981), then cytoplasmic diffusion is an important parameter to monitor independent of junctional membrane permeability, to ascertain whether or not solute diffusion is influenced by cytoplasmic factors. These factors could alter the effective concentration gradient of a solute across a junction and therefore the flux of the solute through the junction. Determining whether molecules follow Fickian diffusion reveals information about the transport process in gap junctions and the role of the cytosol in influencing the free pool of solute within the cell.

In the study, the system studied was the septate median giant axon of the earthworm. The septa lie perpendicular to the long axis of the axon (Stough, 1926) and contain gap junctions (Goodenough, 1975; Makowski et al., 1980) or nexuses (Kensler et al., 1979; Gunther, 1975; and Brink and Dewey, 1978), which act as a partial barrier to dye diffusion. This arrangement allows determination of the junctional membrane permeability and the apparent cytoplasmic diffusion coefficient. The intercellular junctions of the septa are typical of those found in invertebrates with the exception of those in the phylum Arthropoda (Gilula, 1974; and Brink et al., 1981).

Earlier studies on the earthworm system indicated an inverse relationship between molecular weight and junctional permeability with greatly varying apparent cytoplasmic diffusion coefficients. Only short time intervals could be studied because of the constraints of the model used (Crank, 1975; and Brink and Dewey, 1978 and 1980). The model assumed a single permeant surface with axoplasm on either side extending to infinity. Here we develop a model that allows the determination of junctional membrane permeability ($P_j$), plasma membrane permeability ($P_m$) and the apparent axoplasmic diffusion coefficient ($D_x$) at any time interval for a geometry that is equivalent to that of the septate axon. All three parameters were determined for three fluorescent tracers used extensively in studying intercellular communication. These probes were Lucifer Yellow (LY, $1.26 \times 1.4 \times 0.55$ nm), carboxyfluorescein (CFL, $1.26 \times 1.27 \times 0.85$ nm) and dichlorofluorescein (2CFL, $1.23 \times 1.27 \times 0.55$ nm). Dimensions were determined from Corey-Pauling (CPK) models.

**METHODS**

Earthworms (Lumbricus) (Carolina Biological Supply Co., Burlington, NC) were maintained in a mixture of soil, leaves, and mulch at 5°C. Nerve cords were dissected as described by Brink and Barr (1977). A trihydroxymethylamine (Tris) buffered saline (pH = 7.4) was used to bath the earthworms during dissection (Prosser, 1973). The dissected cords were transferred to a saline solution which contained 120 mM Na Acetate, 1 mM K Acetate, 1 mM Ca Acetate, 1 mM Mg Acetate, 5 mM Choline Chloride, and 50 mM Carbocachol, pH = 7.5. All experiments were performed in the acetate saline. Individual septate axons were impaled with a microelectrode and one of the three fluorescent probes, LY, CFL, or 2CFL, was iontophoresed into the cell (Brink and Dewey, 1978).

Hyperpolarizing currents of 30 to 80 nA were applied periodically (once every 50 ms) with a duration of 25 to 30 ms for 10 to 30 min to fill cells of the axon to concentrations of 0.2 to 1 mM. The concentration of a probe inside the axon was estimated by measurement of the fluorescence intensity (Brink, 1983). When the concentration of the fluorescent probe exceeded 2 mM, fluorescence quenching was observed in glass cylinders with inside diameters of 100 µm.

Fig. 1 shows the general experimental setup used to collect and store diffusion data. A grating monochromator in series with the photomultiplier allowed observation of the peak emission of the fluorescence spectra. A $15 \times 50$ µm slit was placed in the optical path before the grating monochromator and photomultiplier to limit the area of measurement and yield high spatial resolution. The source of excitation for the probes was a xenon lamp with a grating monochromator (Farrand Optical Co., Inc., Valhalla, NY). The excitation was delivered to the axons via epi-illumination. The preparation was mounted on a motorized stage (Carl Zeiss, Inc., Thornwood, NY) that could move the field along the long axis of the axon at a constant velocity. For the experiments done in this study the velocity was 100 µm/s. The resultant photomultiplier output then revealed the spatial distribution of the probe within the septate axon. Scans were taken at various time intervals and the photomultiplier output was monitored by a microcomputer (IBM-PC; IBM instruments, Inc., Danbury, CT) via an analog-to-digital (A/D) converter (Data Translation, Marlboro, MA). The data was stored on hard disk for subsequent analysis. The amount of dye iontophoresed from experiment to experiment was varied such that the initial concentration of dye was $\sim 0.2-0.3$ mM or $\sim 1$ mM. The gain of the photomultiplier amplifier was set such that 1 mM = 4 V for each dye. The A/D converter has a voltage window of $\pm 5$ V with a 5 M sensitivity limit. Therefore the apparatus was capable of sensing concentration, as fluorescence intensity, over three orders of magnitude. The lowest concentration detectable was 1 µM. In each figure showing raw data, the vertical scale is given in volts.

The theoretical diffusion coefficients for LY, CFL, and 2CFL in water were calculated from the Einstein-Stokes equation: $D_x = \frac{RT}{6\pi \eta r}$, where $T$ = absolute temperature and $k$ is Boltzmann's constant (see Table I). The viscosity of water ($\eta$) equals 0.01 poise at room temperature (Jacobs, 1967; or Robinson and Stokes, 1968) and $r$ is chosen as one half the widest dimension of the dye molecule as given by CPK models. For this study the diffusion coefficients predicted from the Einstein-Stokes equation ($D_x$) will be used as reference diffusion coefficients for the axon.
The radius was taken as half of the widest dimension of the molecule.

cients for assessment of dye behavior in the axoplasm, as has been the
convention in other studies (Weingart, 1974; Mastro and Keith, 1984).

**THEORY**

A schematic representation of the septate axon is depicted in Fig. 2. The
individual axons have diameters of ~ 80 μm, and axon segments can vary
in length from 500 to 5,000 μm. We consider an axon filled uniformly at
t = 0 with a diffusing solute at concentration C0. The axon interfaces with
two adjacent axons on either side through junctional membranes (P). The
adjacent cells are assumed to extend to an infinite distance and have
adjacent two

and the junctional membranes (P). The
axon filled

Two models for the
diffusion of solute in the septate axon will be considered. The first
assumes the solute diffuses in the cytoplasm in the longitudinal direction
only (one-dimensional model). The second considers both radial and

Longitudinal Diffusion with no Plasma
Membrane Permeability

The junctional membranes are assumed to be equidistant from X = 0 at
some distance A (Fig. 2). By virtue of symmetry either of the regions X ≤
0 or X ≥ 0 can be considered. We will consider only X ≥ 0. The
concentrations of fluorescent probes in regions 0 ≤ X ≤ A and X ≥ A are
denoted C1 and C2, respectively. Let C0 be the initial concentration of the
probe in the injected axon (0 < X < A) at time t = 0. Let D and P denote
the diffusion coefficient and the junctional membrane permeability
respectively. From Fick’s first Law:

\[
D(\partial C_i/\partial X^2) - \partial C_i/\partial t = 0 \\
\]

By symmetry

\[
\partial C_i/\partial X = 0 \\
\]

at X = 0 for all t

\[
D(\partial C_i/\partial X) = P(C_2 - C_1) \text{ at } X = A. \\
\]

Conservation demands that

\[
\partial C_i/\partial X = \partial C_i/\partial X \text{ at } X = A \\
\]

The boundary conditions are:

\[
C_1 = C_0 \text{ for } X ≤ A \text{ at } t = 0 \\
C_2 = 0 \text{ for } X ≥ A \text{ at } t = 0. \\
\]

Applying the methods of Laplace transforms where by definition

\[
\overline{C_i} = \int_0^\infty \exp(-pt)C_i dt, \quad i = 1, 2 \\
\]

and defining \( h = P/D \) and \( q^2 = p/D \), we hypothesize the following relations:

\[
\overline{C_1}(X, p) = A_1(p) x \exp(qX) + B_1(p) x \exp(-qX) + C_0/Dq^2 \\
\overline{C_2}(X, p) = A_2(p) x \exp(qX) + B_2(p) x \exp(-qX), \\
\]

where the barred quantities are the Laplace transform of the corresponding
unbarred quantities. Since \( \overline{C_i} \) must approach zero for large X,
\( A_2(p) = 0 \). Solving in the transform-plane for \( A_i(p) \) and \( B_i(p) \), \( i = 1, 2 \), yields:

\[
\overline{C_1}(X, p) = C_0/Dq^2 + [C_0 x \exp(-qX) + h x \exp(qX)]/(h^2 - qX) \\
\overline{C_2}(X, p) = -(C_0 x \exp(-qX) + h x \exp(qX))/ \exp(qX) + h x \exp(qX). \\
\]

Consideration of the roots of the denominator in the Appendix section 1.1
and subsequent contour integration yields:

\[
C_1(X, t) = -2 x C_0 x (h^2/\pi) \\
\]

\[
\int_0^\infty \cos(uX) x G(u) du \quad \text{ for } X ≤ A, \\
\]

and

\[
C_2(X, t) = -2 x C_0 x (h/\pi) \\
\]

\[
\int_0^\infty \sin(uX) x G(u) du \quad \text{ for } X > A, \\
\]

where u is the integration variable and \( G(u) \) is defined by Eq. 11.

\[
G(u) = \exp( -Du^2 t) x \sin(uA)/[u x [u^2 x \sin(uX)] + \sin(uA) x \cos(uA)] \\
\]

**Longitudinal Diffusion with Finite
Plasma Membrane Permeability**

The equations for the two-dimensional model are:

\[
\partial C_i/\partial t = D[\partial^2 C_i/\partial X^2 + (1/r)\partial C_i/\partial r] \\
\]

\[
+ \partial^2 C_i/\partial r^2 \quad \text{ for } i = 1, 2 \quad \text{(2a)} \\
\]

**TABLE I**

**THEORETICAL DIFFUSION COEFFICIENTS**

<table>
<thead>
<tr>
<th>Dye</th>
<th>( D_\infty )</th>
<th>Radius</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY</td>
<td>3.1 x 10^{-5}</td>
<td>0.7</td>
</tr>
<tr>
<td>CFL</td>
<td>4.0 x 10^{-5}</td>
<td>0.635</td>
</tr>
<tr>
<td>2CLFL</td>
<td>4.0 x 10^{-5}</td>
<td>0.635</td>
</tr>
</tbody>
</table>

**FIGURE 2** Schematic diagram of the axonal model used to analyze the
diffusion data. The total area of the septa was assumed to be a permeable
surface. Arrows indicate longitudinal diffusion through the axoplasm and
junctions (septa) and radial loss across the plasma membrane. The cell
bodies are labeled and note that they are small relative to the axon
segments. They lie within the neuropile of the nerve cord well away from
the axons. Normal injection procedures do not adequately fill the cell
body or its process to cause asymmetry in the longitudinal scans of the
axons.
\[ \frac{\partial C_i}{\partial X} = 0 \] at \( X = 0 \)

(2b)

\[ D(\frac{\partial C_i}{\partial X}) - D(\frac{\partial C_j}{\partial X}) = -P_j(C_1 - C_i) \] at \( X = A \)

(2c)

\[ D(\frac{\partial C_i}{\partial r}) = -P_m \times C_i \] for \( i = 1, 2 \)

at \( r = R; R = \) axon radius.

(2d)

Eqs. 2c and 2d provide the appropriate boundary conditions. Defining Laplace transforms with barred quantities as usual and with \( h_m = P_m/D \) we assert the following hypothesis:

\[ \bar{C}_i(X, p) = 2 \sum_{n=0}^\infty J_0(a_n r) \times \cosh(S_n X) \times A_1(p, a_n) + C_0/Dq^2 \]

(2e)

\[ \bar{C}_j(X, p) = \sum_{n=0}^\infty J_0(a_n r) \times \exp \{(-S_n X) \times B_2(p, a_n)\}. \]

(2f)

Eqs. 2a to 2d show that the hypothesis is valid if \( a_n \) and \( S_n \) satisfy the following equations:

\[ a_n \times J_1(a_n R) - h_m \times J_0(a_n R) = 0 \]

(2c)

and

\[ S_n = (a_n^2 + Dq^2)^{1/2}. \]

(2f)

The roots of Eqs. 2e and 2f are given in Carslaw and Jaeger (p.493, 1959). Substitution into Eqs. 2a to 2d shows that the diffusion of dye was evaluated by the adaptive quadrature method. Accuracy was monitored by checking the known profiles at the initial time \( t = 0 \) to an accuracy of three decimal places for a range of \( D_p, P_r \), and \( P_m \) values. Roots of functional Eqs. 2e and 2f were taken from standard tables and linearly interpolated, a technique valid for the ranges of \( (R/A) \) used. A typical time for the evaluation of an integral is \(-15-20 s \) in compiled BASIC for the IBM-PC (IBM Instruments, Inc.)

RESULTS

For the probes 2CLFL and CFL, \( D_s \) and \( P_j \) remained constant in time, while \( P_m \) declined in time. Five individual diffusion profiles of dichlorofluorescein from a single experiment are shown in Fig. 3. In frames a, c, and d of Fig. 3, \( P_p, D_s, \) or \( P_m \) were varied, respectively, while the other two were held constant, to demonstrate the effect each parameter had on the fitting of the data. In frame a, three curves are displayed with the diffusion profile to demonstrate the effect of \( P_i \) on the diffusion. Both \( D_s \) and \( P_m \) were held constant. The dashed line represents a case where \( P_i = 1 \times 10^{-4} \) and the dotted line \( P_i = 1 \times 10^{-4} \) cm/s. The solid line represents the case that best fits the data where \( P_i = 1 \times 10^{-5} \) cm/s. Fitting was done by eye. The entire surface of the septum was assumed to be accessible by the dye (contact in the plane perpendicular to the long axis of the axons). In Fig. 3 b \( (t = 1,800 s) \), the same parameters that fit the data of 3 a were used. In Fig. 3 c, \( D_s \) was varied while \( P_i \) and \( P_m \) were held constant. Three values of \( D_s \) were used, \( 4 \times 10^{-4} \) (dashed line), \( 4 \times 10^{-5} \) (solid line), and \( 4 \times 10^{-6} \) (dotted line). The best fit for \( D_s \) was \( 4 \times 10^{-7} \) cm²/s, a value an order of magnitude less than would be predicted from the Einstein-Stokes equation for a 1.2 nm diameter molecule.

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In frame 3 a, \( D_s \) was varied while \( P_i \) and \( P_m \) were held constant. Three values of \( D_s \) were used, \( 4 \times 10^{-4} \) (dashed line), \( 4 \times 10^{-5} \) (solid line), and \( 4 \times 10^{-6} \) (dotted line). The best fit for \( D_s \) was \( 4 \times 10^{-7} \) cm²/s, a value an order of magnitude less than would be predicted from the Einstein-Stokes equation for a 1.2 nm diameter molecule. The dashed line represents a \( P_m \) of \( 1.8 \times 10^{-4} \) and the dotted line a value of \( 1.8 \times 10^{-4} \). The best fit for \( D_s \) (solid line) \( t = 1,715 s \) was \( 1.8 \times 10^{-4} \) cm/s. Both \( P_i \) and \( D_s \) were held constant at \( 1 \times 10^{-5} \) and \( 4 \times 10^{-7} \) respectively. \( P_m \) declined from \( 2.3 \times 10^{-7} \) cm/s to \( 1.8 \times 10^{-7} \) cm/s between 3,600 s and 7,150 s. In all five experiments analyzed (Table II) for the dye 2CLFL, \( P_m \) showed a decline in time while the other two parameters remained constant. In all cases at least three and as many as six records were analyzed from each experiment. The model assumes that all dye that leaks out of the plasma membrane is washed away so that the only decline in the radial concentration gradient comes from the decline of dye concentration in the axoplasm. The reduction of \( P_m \) with time suggests that the dye is not washing away to infinite dilution but that significant amounts are present in the periaxial space and loose myelin covering of the axon. If dye fading with light exposure was a significant contributor to apparent dye loss in the axon, then the model would...
predict an increase in plasma membrane permeability with time. This was not found in any of the records analyzed but rather a decline in $P_m$ was observed. Changes in radius of the axon with time will also affect $P_m$. Therefore the diameter of the injected cells was monitored during the experiments. No diameter changes were observed. In the case of Fig. 3 the axon diameter would have to narrow from 80 to 60 μm. Fig. 3 e shows that the dye was no longer detectable by the photomultiplier 51,000 s after injection, and the model predicted that the dye concentration in the axon was < 0.001% of the original concentration in the injected cell using the fit parameters of 3 d. If a large portion of the dye had been bound or trapped in an irreversible fashion within the axon some fluorescence would still be expected to be observed within the axon at the 50,000 s mark.

Five diffusion profiles of the dye carboxyfluorescein are shown from a single experiment in Fig. 4 with the best fit for each spacial distribution. Both $D_A$ and $P_j$ remained constant (Table II) over the time interval studied, but $P_m$ declined 37% over a 17,100 s interval. This decline is presumably due to the accumulation of dye in the periaxonal space as described for 2CLFL. Once again no change in the radius of the axon was observed. Trace amounts of CFL were detectable in axons 150,000 s after injection as the insert in frame 4 e shows. The residual fluorescence of CFL indicates a small fraction of dye may irreversibly bind to components of the axoplasm and axolemma or have a very slow dissociation rate with those components.

In Table II the ratios of $P_j$, $D_A$, and $P_m$ for 2CLFL and CFL are given. The $P_j$ mean ratio for 2CLFL/CFL was 19.7 with a range of 4.7 to 73, and the $D_A$ ratio was 1.08. The range over which $D_A$ varied for both dyes was 2.8 and the data sets overlapped almost completely. The $D_A$ ratio indicates that 2CLFL and CFL are affected similarly by axoplasmic factors. The $P_m$ ratio followed the same trend as the $P_j$ with a mean ratio of ~ 5:1.

Four diffusion profiles of LY from a single experiment are shown in Fig. 5. Unlike the two previous dyes, $D_A$, $P_j$, and $P_m$ for LY all declined with time. $D_A$ declined 70%, $P_j$ declined 88%, and $P_m$ was reduced ~50% over a 28,000 s interval. The $D_A$ for LY started out quite high (2.0 × 10^{-6} cm²/s); a value close to the value expected from diffusion in water. The reduction of $D_A$ with time indicated that the dye was binding or complexing in some form with axoplasmic elements as Stewart (1978) has already suggested. Fig. 6 shows another experiment where significant

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**Figure 3** Fluorometric data for 2CLFL. 3 a shows diffusion 600 s after injection of the dye was terminated. The solid smooth line is the best fit by eye for the data. The dashed and dotted lines represent fits where $P_j$ was varied while $D_A$ and $P_m$ were held constant. 3 b shows the best fit at 1,800 s. 3 c shows the best fit at 3,600 s and the effect of varying $D_A$. 3 d shows the effect of varying $P_m$. In 3 e the data show that no dye is present at 50,000 s and the model predicted a concentration in the injected cell of 0.001% (4 V = 1 mM).
TABLE II
DIFFUSION CONSTANTS FOR DICHLOROFLUORESCIN AND CARBOXYFLUORESCIN

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$D_a \times 10^4$</th>
<th>$P_i \times 10^5$</th>
<th>$P_m \times 10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichlorofluorescein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>050484 ($T = 10,400$)</td>
<td>0.70</td>
<td>3.0</td>
<td>5.1-2.0</td>
</tr>
<tr>
<td>051184 ($T = 8,800$)</td>
<td>0.80</td>
<td>1.1</td>
<td>4.0-1.8</td>
</tr>
<tr>
<td>062184 ($T = 11,000$)</td>
<td>0.35</td>
<td>1.2</td>
<td>4.1-1.9</td>
</tr>
<tr>
<td>070284 ($T = 51,000$)</td>
<td>0.40</td>
<td>1.0</td>
<td>2.3-1.8</td>
</tr>
<tr>
<td>072784 ($T = 1,400$)</td>
<td>0.42</td>
<td>2.2</td>
<td>2.0-1.4</td>
</tr>
<tr>
<td>Average</td>
<td>$5.3 \times 10^{-7}$</td>
<td>$1.7 \times 10^{-5}$</td>
<td>$D_a = 4.0 \times 10^{-6} \text{ cm}^2/\text{s}^\dagger$</td>
</tr>
<tr>
<td>Carboxyfluorescein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>061384 ($T = 20,600$)</td>
<td>0.7</td>
<td>0.210</td>
<td>0.2-0.1</td>
</tr>
<tr>
<td>070684 ($T = 17,000$)</td>
<td>0.5</td>
<td>0.050</td>
<td>1.6-1.0</td>
</tr>
<tr>
<td>072084 ($T = 23,000$)</td>
<td>0.42</td>
<td>0.070</td>
<td>1.0-0.7</td>
</tr>
<tr>
<td>080184 ($T = 12,800$)</td>
<td>0.60</td>
<td>0.041</td>
<td>0.9-0.6</td>
</tr>
<tr>
<td>091884 ($T = 105,000$)</td>
<td>0.25</td>
<td>0.061</td>
<td>0.4-0.2</td>
</tr>
<tr>
<td>Average</td>
<td>$4.9 \times 10^{-7}$</td>
<td>$8.6 \times 10^{-7}$</td>
<td>$D_a = 4.0 \times 10^{-6} \text{ cm}^2/\text{s}^\dagger$</td>
</tr>
</tbody>
</table>

$P_{2CFL}/P_{CFL}$ average 20; range of variation 4.7 to 73. $D_{2CFL}/D_{CFL}$ average 1.08. $P_{2CFL}/P_{CFL} \sim 5$.

$\dagger$ Represents the number of scans analyzed in an individual experiment.

$\ddagger$ $T$ is the time in seconds of the last scan made in any experiment.

$\$ Diffusion coefficients computed from the Einstein-Stokes equation.

$||4.7$ is the ratio for 070284 and 061384, 73 compares 050484 and 080184.

Amounts of LY were still present after 71,000 s, indicating binding or some other form of retardation of movement of the dye was taking place. $D_a$ was reduced in this record from $3 \times 10^{-6}$ to $7 \times 10^{-8} \text{ cm}^2/\text{s}$, a 50-fold reduction. $P_i$ fell from $3 \times 10^{-6}$ to $6.5 \times 10^{-7} \text{ cm/s}$ over the same interval, an 80% reduction. Fig. 7 shows the changes in $D_a$ and $P_i$ with time for LY for four experiments. Each point (triangle, circle, or square) represents the best fit for a diffusion profile. In each experiment between three and five records were analyzed. In all cases $D_a$ was reduced with time. $P_i$ declined with time for LY in all of the experiments as Fig. 7b shows. $P_m$ mirrored the decline of

FIGURE 4 Analysis CFL in a similar fashion to that of Fig. 3 where only the best fits are shown. The insert of 4e shows another CFL injection which shows residual fluorescence at 150,000 s (4 V = 1 mM).
**FIGURE 5** Fluorometric data for LY. In all cases, $D$, $P_i$, and $P_m$ decreased with increased time after injection. The dye concentration was in the 0.4-0.5 mM range ($4 V = 1$ mM).

**FIGURE 6** The same as Fig. 5 but the initial concentration was 0.9 mM and the diffusion was monitored for a much longer time.

$P_i$ with time. A decline in $P_i$ would be expected if the pool of freely diffusing LY declined due to binding, therefore decreasing the number of molecules that can diffuse through the junction.

In all of the diffusion profiles the fluorescence intensity varied as much as 20-30% within the injected axon where the model predicted smooth curves. Fluctuations of fluorescence intensity along the axon were caused by the fact that the axon diameter varied as much as 50% from one region to the next. Fig. 8 shows a diffusion profile for the dye CFL and a fluorescence micrograph of the same cell. Assuming an even distribution of dye within the axon, those areas of smaller diameter will present less fluorescence intensity to the slit of the photomultiplier than regions of greater diameter, thus producing oscillations in
the fluorescence along the axon. Because of the fluorescence fluctuations inherent to all records, the effect of radius change on the model was assessed to test whether $D_a$ and $P_j$ were independent of radius. Neither $P_j$ nor $D_a$ were influenced by increasing or decreasing axon diameter while changing $P_m$ proportionately. In all cases $P_m$ had to be altered linearly with changes in diameter to fit the data of Fig. 3c, indicating that $P_m$ was affected by the surface-to-volume ratio of the cell. Thus $P_j$ and $D_a$ are insensitive to changes in radius. In Figs. 3 and 4 the longitudinal waveform remains relatively constant from scan to scan as Fig. 8 suggests. But in Figs. 5 and 6, which depict LY diffusion, the longitudinal waveform does not remain constant for all times (i.e., peaks and, most notably, valleys appear and disappear). This variation in the LY profiles is most probably related to localized binding (sequestration) to cytosolic and membrane-bound elements. Localized regions either salt out the dye, rendering it nonfluorescent or possibly bind or sequester the dye, decreasing its fluorescence efficiency thus causing a decline in fluorescence intensity. With time, more dye diffuses in from adjacent regions, causing an increase in intensity. Note that CFL and 2CLFL are not affected in this way.

**FIGURE 7** (a) Semi-log plot showing the decline of $D_a$ vs. time for LY from four individual experiments. (b) Semi-log plot of $P_j$ vs. time for the same experiments.

The apparent axoplasmic diffusion coefficients for CFL and 2CLFL are 8 and 7.4 times less than that predicted for the diffusion coefficients in water. The reduction can be attributed either to axoplasmic viscosity, tortuosity factors, binding or sequestration of the dyes to axoplasmic components, or a combination of the four. Some aspects of dye behavior in the axoplasm can be indirectly ascertained by making assumptions about viscosity, binding, and tortuosity. Sequestration is not dealt with separately but is considered a component of binding. If it is assumed that the viscosity of the axoplasm is approximately two times that of water (Mastro and Keith, 1984; Robinson and Baker, 1979), then the apparent viscosity of the axoplasm would be in the range of 0.02 to 0.03 poise at 20°C, causing $D_a$ to fall near $2 \times 10^{-4}$ cm²/s for the three probes. This leaves a factor of 3 to 4.5 reduction in $D_a$ for both CFL and 2CLFL, which must arise from either binding or the tortuosity imposed by the cytoskeleton and cellular organelles. The tortuosity factor causes a reduction of the effective cross-sectional area in any plane into which a dye molecule can move (translational motion) in the form of immobile objects that impede the “random walk” of a molecule. A difference in the rotational and translational motion would be expected if tortuosity were a significant factor in determining the value of the diffusion coefficient for a molecule. In cultured mammalian cells this was not found to be the case (Mastro and Keith, 1984), thus tortuosity is probably not a significant factor in influencing $D_a$. If tortuosity were the major factor causing the reduction of

**DISCUSSION**

**FIGURE 8** Correlation between fluorescence intensity distribution and axon diameter. An axon was injected with CFL and scanned (trace in the lower portion of the figure). The fluorescent axon was then photographed on a Zeiss Universal Microscope. Note that with increased diameter the intensity increased. See the text for further explanation. Photomultiplier slit was 15 \( \times \) 50 μm.
dye diffusion, then cytoskeletal and organelle density would be such that ~60–90% of the cross-sectional area would be occupied by them. The last factor that can cause a reduction in $D_i$ is binding of dye molecules to components of the axoplasm and axolemma. The effects of nonspecific (nonsaturatable) reversible binding on diffusion can be assessed using the following:

$$D_b = \left( \frac{M_f}{M_t} \right) \times D_i,$$

(3)

where $M_t$ is the mole fraction that is free in the axoplasm; $M_i$ is the total molar concentration in the axon such that $M_t = M_f + M_b$; $M_b$ is the mole fraction bound; $D_f$ is the diffusion coefficient for a solute in the axoplasm unimpeded by binding. The diffusion coefficient of the bound solute ($M_b$) is $D_b$. This analysis requires that $D_b \ll D_i$ or $D_b = 0$. Thus the bound solute is considered to have no translational motion.

For the case of saturable binding, if it is assumed that the reaction is irreversible or that the rate of dissociation is orders of magnitude slower than the rate of association, then Eq. 3.1 can be used to assess the mole fraction bound. Once again $D_b$ must be much smaller than $D_i$ ($D_b \ll D_i$). This assumption does not appear to be unreasonable when the Lf data is considered. In the case of Lf a significant residual fluorescence remains and can be visualized days after injection.

If $D_b$ approximates $D_i$ then $dC/dx$ will have more than one diffusion profile along the $x$ axis and Eq. 3.1 will not hold. Two illustrations of this are shown in Figs. 9 and 10. Fig. 9 is the case of nonspecific binding where $M_b = M_f$ and $D_f (2 \times 10^{-6})$ is 10-fold greater than $D_b (2 \times 10^{-7})$. Note that in the adjacent cells two distinct diffusion profiles can be seen. While the data do not suggest this kind of behavior they cannot unambiguously eliminate this possibility. The diffusion profile of Fig. 10 was generated using the diffusion model. In both cases $C_0$ was set at 0.5 and the two resultant curves were summed. Fig. 10 illustrates the case where a saturable binding site with a $D_b$ 10-fold less than $D_i$ must first be loaded due to its high affinity for the solute. The concentration of the binding site is one-tenth that of the initial dye concentration. The diffusion profiles in the adjacent cells show first the diffusion profile determined by the slowly diffusing binding sites; then the unimpeded mole fraction begins to dominate the diffusion profile. The diffusion model was used to generate the curves by setting $C_0$ for the bound solute at 0.1 and $C_0$ for the freely diffusing solute at 0.9. The diffusion profile of the bound component was plotted as the leading edge of the profile. It was assumed that the association rate is much greater than the dissociation rate. The data did not show this kind of behavior.

If as already stated it is assumed that $D_b \ll D_i$, then Eq. 3 can be used to estimate the amount of bound dye. The fact that both CFL and 2CLFL do not leave significant residual fluorescence in the injected and adjacent cells, and $D_b$ and $P_f$ remain constant for CFL and 2CLFL argues strongly for nonspecific binding of these two molecules. If the viscosity of the axoplasm is assumed to be that of water, then the percent of bound dye for CFL is 88% (Eq. 3) or 76% with a viscosity two times that of water (Mastro and Keith, 1984). For 2CLFL the bound fractions would be 85% or 73%, making the same assumptions about viscosity as were made for CFL. These estimates of bound dye fraction cannot account entirely for the retarded value of $D_f$ relative to water but do demonstrate to a first approximation that nonspecific binding is a major determinant in establishing the free pool for these dyes. In Table III $D_b/D_s$ and percent dye bound are given for all three dyes. The computed $D_b$ for LY at short time intervals is reduced 45% relative to $D_s$. This reduction is consistent with the notion that at least initially, LY is slowed by cell water viscosity only. With time $D_b$ declines (Fig. 7), indicating that the dye is effectively bound to components of the axoplasm and axolemma; Eq. 3 indicates that the binding is ~95%
TABLE III
COMPARISON OF DIFFUSION COEFFICIENTS IN AXOPLASM AND WATER

<table>
<thead>
<tr>
<th>Dye</th>
<th>( D_0/D_\infty )</th>
<th>% Bound*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFL</td>
<td>0.12</td>
<td>76</td>
</tr>
<tr>
<td>LY</td>
<td>0.55( \dagger )</td>
<td>0</td>
</tr>
<tr>
<td>2CLFL</td>
<td>0.02( \dagger )</td>
<td>95</td>
</tr>
<tr>
<td>CFL</td>
<td>0.13</td>
<td>73</td>
</tr>
</tbody>
</table>

*% bound based on an axoplasmic viscosity 2 times that of water.
\( \dagger \) For short time intervals after injection (<1,500 s). \( D_0 \) and \( D_\infty \) values for LY are taken as the average value from the data of Fig. 7.
\( \dagger \) For long time intervals (>20,000 s).

effective. Whether the binding is high affinity or low affinity it appears to be irreversible for LY. The maximum and minimum values of \( D_0/D_\infty \) for LY are shown in Table III, illustrating the decline of \( D_\infty \) with time. The \( D_0/D_\infty \) ratio for the other two molecules remained constant in time.

The ratio of \( D_0/2CLFL/D_0CFL \) was 1.08 (Table II). This implies, but does not unequivocally mean, that the two dye molecules experience the same impediments to diffusion in the intracellular compartment(s). The ratio of \( D_0/LY/D_02CLFL \) or \( D_0CFL \) was \( \sim 4 \), using \( D_0 \) values of LY for short time intervals. This indicates that LY is not being affected by the same axoplasmic components as are CFL and 2CLFL (e.g., nonspecific binding).

Because CFL and 2CLFL have similar values of \( D_\infty \), a comparison of junctional membrane permeability can be made. CFL has a \( P_\text{r} \) that is reduced by a factor of 4.7 to 73 relative to that of 2CLFL. Both size and charge play an important role as determinants of solute mobility within the junctional channels (Loewenstein, 1981; and Brink and Dewey, 1980). CFL has dimensions of \( 1.26 \times 1.27 \times 0.85 \) nm and has two carboxyl groups with pKs in the range of 3–4. 2CLFL has dimensions of \( 1.23 \times 1.27 \times 0.55 \) nm with only one carboxyl group. The only size difference is the narrowest dimension. If the channel is assumed to have a more or less spherical orifice with its narrowest dimension being \( \sim 1.5 \) nm (Makowski et al., 1984; and Loewenstein, 1981), then the limiting dimensions for CFL and 2CLFL are 1.26 and 1.23 nm respectively. The lack of dimensional disparity strongly suggests a role for charge not only on the solute but also within the junctional channel in determining the permeability of the junctional membrane. These results are consistent with the concept of fixed charge groups within or at the entrance of the intercellular channel (Brink and Dewey, 1980 and Flagg-Newton et al., 1979) which in effect, by virtue of field effects, present channel diameters of different sizes to solutes depending on the surface charge density of the solute molecule diffusing through the junctional channel.

Experiments using heavy water exchange show an activation energy difference between \( D_2O \) saline and \( H_2O \) of 0.8–1.0 kcal/mol for both dye diffusion (Brink, 1983) and conductance (Brink et al., 1984) indicating that there is some form of hydrogen or deuterium bonding between solute and channel. The earthworm septal junctions do not show transjunctional voltage-sensitive conductances as do amphibian and teleost blastomeres (Spray et al., 1981 and 1984) but do show pH sensitive conductances (Verselis and Brink, 1984). In the earthworm the pH sensitivity is of questionable physiological significance but does imply that titratable charge groups near or within the channel are present. These proposed charge groups and/or gates could be responsible via field effects for the reduced \( P_\text{r} \) of CFL relative to 2CLFL.

The binding of the dyes to the axoplasm reduces the effective free concentration of the dye in the cell, and since the model assumes all the injected dye is free to diffuse, the computed values of \( P_\text{r} \) are suppressed. Correcting for the binding (Eq. 3) elevates \( P_\text{r} \) ~ eightfold for CFL and 2CLFL. Thus \( P_\text{r} \) would be \( \sim 1 \times 10^{-4} \text{ cm/s for 2CLFL} \) and \( 6 \times 10^{-6} \text{ cm/s for CFL. No such correction need be made for LY since, at least initially, the dye appears to diffuse in the axoplasm with viscosity as the major determinant of } \( D_0 \).

In conclusion, the dyes LY, CFL and 2CLFL were shown to diffuse nonideally in the axoplasm of the septate axon. In the case of CFL and 2CLFL \( D_\infty \) was reduced by a factor of 8 while LY behavior was indicative of a slow binding process or sequestration. Note that LY in K+ salt solutions precipitates as a K salt and is nonfluorescent in that form. But inside cells it is quite fluorescent and at least in the earthworm appears to be nonotoxic even with exposure to light (Brink et al., 1984). The slow decline in \( D_\infty \), \( P_\text{r} \), and \( P_\infty \) may be an indication of a slow salting out of dye with K+. Finally the data suggests that both size and charge are important in determining the diffusion rate of a molecule in the intercellular channel and that, to fully understand the rate of transfer across the junction of a solute molecule, the behavior of the solute within the cytosol of the cell must be assessed. Diffusion modeling of the sort utilized in this study also has potential significance in the modeling of embryonic systems where morphogens are thought to play a significant role in cellular differentiation.

APPENDIX

Section 1.1

For the purposes of contour integration we need to know if \( C_1 \) and \( C_2 \) are multivalued. The roots of the denominator of \( C_1 \) and \( C_2 \) in the complex \( p \)-plane must also be evaluated. Fortunately, both \( C_1 \) and \( C_2 \) share the same denominator, which is:

\[
Dq^2 [q \times \sin h(qA) + h \times \exp (qA)] \quad (A1)
\]

The square root of \( p \) defines the denominator as multivalued. To make it single valued we define a branch cut in the complex \( p \)-plane extending from \( p = 0 \) to \( p = -\infty \) along the real axis. The roots of the denominator are evaluated as follows: One obvious root is \( p = Dq^2 \sim 0 \). The possible roots of \( [q \times \sin h(qA) + h \times \exp (qA)] \) must also be considered. If \( q \) is purely imaginary such that \( -iA \sim \text{then Eq. A1 gives} \exp (2 \times iA) = iA/(iA + 2A) \). This yields the modulus \( \eta^2 = \eta^2 + 4A^2 \), which excludes
imaginary roots. It can also be shown that $q$ cannot be a complex root of the form $(e + in)$ by the standard method (Carslaw and Jaeger 1959, p. 323). For this consider the function $U$, defined as follows:

$$U_i = \exp(-bA) \times \cosh(bx) \quad \text{for} \quad 0 < x < A$$
$$U_i = -(1/h) \times [x \times \cosh(bA) + b \times \sinh(bA)] \times \exp(-b\lambda) \quad \text{for} \quad x > A,$$

where $b$ satisfies Eq. A2:

$$b \times \sinh(bA) + h \times \exp(bA) = 0. \tag{A2}$$

Then it can be shown that: $dU_i/dx^2 - bU_i = 0 \quad \text{for} \quad x \neq 0; U_i = 0 \quad \text{as} \quad x \to \infty$; $dU_i/dx = b \times \exp(-bA) \times \sinh(b(A - x))$ and $dU_i/dx = h(U_i - U_2) \quad \text{at} \quad x = A$; $dU_i/dx = -dU_i/dx \quad \text{at} \quad x = -A$. Let $a$ and $b$ be two different roots of Eq. A2 and $U_i$ and $V_i$ be the corresponding quantities. Then it can be proved (Carslaw and Jaeger, 1959) that:

$$(b^2 - a^2) \left[ \int_0^x (U_i + V_i)dx + \int_x^\infty (U_i - V_i)dx \right] = 0. \tag{A3}$$

If $a$ and $b$ are complex conjugates of the form $e \pm in$ then $(b^2 - a^2)$ cannot equal zero but since $U_i$, $V_i$, and $U_i + V_i$ are complex pairs, the term in square brackets is positive (Eq. A3) and is a contradiction. Thus all roots of $h \times \exp(bA) + b \times \sinh(bA)$ are real. But since $b \times \sinh(bA) > 0 \quad \text{for} \quad a$ and $b \times \exp(bA) > 0 \quad \text{for} \quad \text{all real} \quad b$, we conclude that $h \times \exp(bA) + b \times \sinh(bA)$ has no roots at all, thus allowing us to invoke the inversion theorem. The contour of integration is chosen to avoid the pole at $p = 0$, and the branch cut along the negative real axis. Contour integration then yields Eqs. 1h and 11.

**Section 1.2**

In the Appendix, section 1.1, it can be shown that the denominator $[S_a \times \sinh(S_bA) + h \times \exp(S_bA)]$ does not have any roots at all (Carslaw and Jaeger, 1959). It is also necessary to determine if $A_1$ and $B_1$ are multivalued. There is a root due to $p = Dq^2 + a_0 = 0$ and $C_1$ and $C_2$ both involve $S_a = (Dq^2 + a_0^2)^{1/2}$. To make it single valued we define a branch cut from the root of $S_a = 0$, therefore from $Dq^2 + a_0^2 = 0$ to $\infty$ along the real axis. The contour of integration is now chosen to avoid the pole at $p = 0$ and the branch cut from $-a_0^2$ to $\infty$ along the negative real axis.

The authors would like to thank Drs. Jiaslow and Kasianowicz for their helpful and careful discussions.

This work was supported by National Institutes of Health grant GM 29405 and National Science Foundation grant 83-14295.

Received for publication 26 December 1984 and in final form 8 April 1985.

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