Nexal Membrane Permeability to Anions

PETER R. BRINK and MAYNARD M. DEWEY

From the Department of Anatomical Sciences, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11794

ABSTRACT The permeability of the septa of the earthworm in the median axon has been calculated for the anions fluorescein and its halogen derivatives. The values ranged from $5.4 \times 10^{-5}$ to $4 \times 10^{-6}$ cm/s. Previously, the septa had been shown to contain nexuses. By using freeze-fracture material, the surface area of nexus on the septal membranes was determined to be 4.5%, very similar to the percentage of nexus in the intercalated disk of mammalian myocardium. Plasma membrane permeability to these dyes was also calculated and shown to be much less than that of the septal membranes. In addition, an estimate of cytoplasmic binding for each dye was made, and most dyes showed little or no binding with the exception of aminofluorescein.

Early electron microscope observations of thin sections of many tissues revealed specialized junctional areas of plasma membranes called nexuses which were thought to be low resistance junctions (Dewey and Barr, 1964; Barr et al., 1965; Barr et al., 1968). Similar structures have been described in many other tissues (Robertson, 1963; Martin and Pilar, 1963; Farquhar and Palade, 1963; Pappas and Bennett, 1966).

The work of Barr et al. (1965, 1968) showed that smooth muscle and myocardial cells were electrically continuous. The structure found responsible for current flow from cell to cell was the nexus. Thus, smooth muscle and myocardium can be thought of as being electrical syncytia. In addition, certain neurons and epithelia are also electrically coupled (Furshpan and Potter, 1959; Bennett et al., 1967; Pappas et al., 1971; Loewenstein, 1975).

Evidence has also been compiled to show biochemical cooperation between cells joined by nexuses (Subak-Sharpe et al., 1969; Gilula et al., 1972; Cox et al., 1974; Gilula, 1974). Before cell systems can be called biochemical syncytia, it must be shown that nexal membranes are not absolute barriers to molecular diffusion between cells or that molecules important to the biochemical integration of a tissue can easily diffuse across the nexal membranes. Although the size of molecules that can get through a nexal membrane has been estimated (Weidmann, 1966; Loewenstein, 1975), only a few estimates of nexal membrane permeability to various probes have been made in mammalian myocardium (Weidmann, 1966; Imanaga, 1974; Weingart, 1974; Pollack, 1976).

The purpose of this work was to measure nexal membrane surface area in septal membranes of the giant axon of earthworm and the permeability of the nexus to various fluorescent dyes varying in mol wt from 330 to 835.

The dyes could be excited with a tungsten source. Knowledge of nexal...
membrane permeability to the various dyes should allow reasonable estimates of the rate at which biochemical compounds diffuse across a nexus and establish a limit of molecular size that can traverse the nexus.

**METHODS**

*Animals, Saline, and Dissection*

Earthworms were purchased from West Jersey Biological (Wenonah, N. J.), and maintained at 4°C in a mixture of mulch and soil. Saline was prepared as described by Prosser and Brown (1950) with a trihydroxymethylamine (Tris) buffer (pH 7.2). Osmolarity of the saline (245 mosmol) was measured by an Advanced Digimatic osmometer (Advanced Instruments, Inc., Needham Heights, Mass.). Nerve cords were dissected as described by Kao and Grundfest (1957) and placed in a chamber as described by Brink and Barr (1974, 1977). Stimulating electrodes were placed at both ends of the nerve cord so that action potentials could be elicited from both directions.

*Freeze-Fracture*

Nerve cords were fixed in 1.5% glutaraldehyde and 0.1 M Na cacodylate buffer for 4 h followed by a 12-h exposure to 40% glycerol in 0.1 M Na cacodylate buffer. 3-mm lengths of nerve cord, dorsal side up, were rapidly frozen in liquid Freon 22 (Virginia Chemicals Inc., Portsmouth, Va.) and liquid nitrogen and were fractured and replicated at -115°C (pressure, $5 \times 10^{-6}$ t) on a Balzers BF 300 freeze-etch device (Balzers High Vacuum Corp., Santa Ana, Calif.). Platinum carbon shadowing by an electron gun was employed within 2-3 s of cleavage and was followed by a 20-nm coating of carbon onto the replica. Replicas were cleaned with Clorox (The Clorox Company, Oakland, Calif.), rinsed in distilled water, and picked up on slot grids (Formvar coated, Monsanto Company, St. Louis, Mo.). Axons were easily identified because they are myelinated and large (Bullock and Horridge, 1965). The septa traverse the cytoplasm and are easily distinguished in the midst of large cytoplasmic expanses.

Freeze-fractured septa were photographed, and the total area of septal membrane was calculated using an automated planimeter (Numonics Corp., Landsdale, Pa.). It was assumed the surface was flat. In each micrograph the total number of particles or pits were counted so an estimate of total nexus surface area could be made.

All electron microscope observations were made on an Hitachi 12 electron microscope (Hitachi Ltd., Tokyo, Japan), and all images are printed as positive images.

*Dyes and Their Injection*

Fluorescein (mol wt 333), dichlorofluorescein (mol wt 401), dibromofluorescein (mol wt 490), diiodofluorescein (mol wt 588), tetrabromofluorescein (mol wt 652), tetraiodofluorescein (mol wt 835), and aminofluorescein (mol wt 342) were purchased from Eastman Kodak Co. (Rochester, N.Y.). Each dye was dissolved in 1.5 M KOH so that its final concentration was 0.3-0.4 M. The solutions were then slowly titrated with $H_2PO_4$ to pH 7.6-7.8.

Microelectrodes were made on a David Kopf vertical puller (David Kopf Instruments Inc., Tujunga, Calif.). Their resistance ranged from 15 to 50 Mohms regardless of the dye used. The electrodes were connected to a WPI amplifier (model 4M-A, W-P Instruments, Inc., New Haven, Conn.), which in turn had input to a Tektronix D10 oscilloscope (Tektronix, Inc., Beaverton, Oreg.). A Tektronix 160 series was used for pulse generation. Once a dye electrode was inserted into an axon it was used to monitor the resting potential and action potential of the axon. Fluorescein, dichlorofluorescein,
and dibromofluorescein were iontophoresed by applying hyperpolarizing pulses of 20–
30 nA, with a 100-ms duration every 120 ms. The iontophoretic period lasted 30 min.
Aminofluorescein was iontophoresed by application of a depolarizing pulse with the
same amplitude, frequency, and duration as fluorescein. Diodo fluorescein, tetrabromo-
fluorescein, and tetraiodofluorescein were iontophoresed by applying hyperpolarizing
pulses of 10–20 nA with 100-ms duration every 120 ms for 60–80 mins.

Permeability Calculation

Fluorescence was monitored by a Zeiss ultraphot microscope (Carl Zeiss, Inc., New
York). A tungsten source was used with a BG12 excitor filter for dye excitation. A Zeiss
barrier filter (530 nm) was used to observe dye fluorescence. The absorption spectra
varied from 489 nm for aminofluorescein to 516 nm for tetrabromofluorescein. The
emission spectra varied from 535 to 565 nm (Berlman, 1971).

Within 1–2 min after the termination of the dye injection period, injected cells were
photographed with an exposure of 40 s. Preparations were kept in total darkness, except
during photographic periods. 40-s exposures were made at varying time intervals.

The concentration of the dye in the injected cells was estimated by microdensitometric
and spectrophotometric methods. Both methods gave similar results. First, glass tubes
were drawn out so that the inside diameters were 50–100 μm (the outside diameter 250
μm) and were filled with dye concentrations ranging from 0.1–1.0 mM. The filled tubes
were photographed under the same conditions as the injected cells. Microdensitometric
scans of the negatives allowed a standard against which the density of the negative of the
injected cell could be compared and concentration could be estimated (Fig. 1). The
greater the dye concentration, the greater the density; therefore, the greater the peak
height given by the microdensitometric scan. The peaks were measured in centimeters
from the base line and plotted against the concentration of the dye. Scans were made
across the negatives perpendicular to the long axis of the dye-filled tubes. Comparison of
the peaks of injected cells with those in Fig. 1 allowed an estimate of the dye concentration
in the cell. There was a great deal of variation in these measurements but it did allow
estimates of cellular dye concentrations.

A second method involved iontophoresis of a dye into a 300-μl cuvette (Precision Cells
Inc., Hicksville, N. Y.) containing 0.2 M KCl. The iontophoretic period was the same as
that used for dye injection of the axon. A dual beam spectrophotometer (Amicon DW-
2UV-VIS, Amicon Corp., Lexington, Mass.) was used to determine the concentration of
dye in the cuvette. The absorption peak observed was compared with a standard of
absorption vs. dye concentration made from known concentrations (Fig. 2).

The volume of the injected cell was calculated from the cell diameter and length as
revealed by the dye injection assuming the cell approximated a cylinder. Because the
iontophoretic period for a specific dye was the same for the axon and the cuvette,
multiplying the ratio of the cell volume to cuvette volume by the concentration of dye in
the cuvette as calculated spectrophotometrically, the concentration of dye in the cell was
determined.

Microdensitometric tracings (Joyce, Loebl model III, Joyce, Loebl and Co., Ltd.,
Gateshead-on-Tyne, England) were made of negatives taken just after termination of the
dye injection period (t=1 min). Negatives taken at various time intervals after termination
of dye injection were also scanned at varying time intervals. The area under the curves
obtained from the microdensitometric scans decreased slightly with increasing time. This
loss was thought to be due to dye efflux across the plasma membrane and quenching.
The shift in the area from one time to another indicated that dye had diffused across the
septa. Areas under the curves were calculated with an automated planimeter (Numonics
Corp.). Negatives of dye injections taken with the ultraphot microscope had magnifica-
tions of \( \times 39 \). The window width was 5 mm and the slit width was 1 mm on the microdensitometer.

The area under the curve was proportional to the dye concentration in that cell. Therefore, by calculating the shift in area under the curve of the injected cell to the adjacent cells for a specific time interval, the flux of a dye across each septal membrane could be computed. The amount of dye that traversed a septum in a given time interval multiplied by cell volume gave the number of moles that diffused across the septum. The mole number was divided by the surface area of nexus on the septal membrane times the

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**Figure 1.** Tubes (inside diameter \( \leq 100 \mu m \)) filled with various dye concentrations were photographed in the same manner as injected cells. Microdensitometric scans show an approximate proportionality between concentration and peak height. The peak heights were plotted (centimeters) against concentration. Microdensitometric scans were made from the negatives of the tubes. The scans were made perpendicular to the long axis of the tubes. The peak density gave the largest deviation from the base line and was measured in centimeters to give the peak height. FL = fluorescein, 2CLFL = dichlorofluorescein, 2BRFL = dibromofluorescein, 2IFL = diiodofluorescein, and 4BRFL = tetrabromofluorescein. Concentrations of the various dyes ranged from 0.1 to 1.0 mM.
time interval which gave flux (moles/centimeters²·second). Nexus surface area was estimated to be 4.5% of the septum from freeze-fracture data given in Table I. The septa traverse the axon at an oblique angle of 140° with little folding of the septal membranes (Stough, 1930; Gunther, 1971, 1975). Therefore, the total septal membrane has been estimated as being approximately 2-2.5 times the cross-sectional area of the axon. 4.5% of that value was taken as the conducting surface of the septum. Dividing the flux by the differences in the dye concentration of the injected and the adjacent cell gives nexal membrane permeability (Katz, 1966). The average dye concentration was used for the computations. Permeability values were calculated at varying time intervals on any one preparation.

\[
P_n = \frac{M_s}{C},
\]

where \(P_n\) = nexal membrane permeability (the nexal membrane permeability equals the septal membrane permeability); \(M_s\) = flux (moles/cm²·s), the number of moles which traverse the nexal membranes of the septal membrane over a given time interval; and \(C\) = the concentration of dye in the injected cell minus the concentration in the adjacent cell.
For the movement of charged molecules in a membrane with an electrical field, the flux of a molecule is dependent on both concentration and the electrical field. If it is assumed that \( \frac{dV_m}{dx} \) is constant through the membrane, then the constant field equation (Goldman, 1943; Hodgkin and Katz, 1949) describing the efflux of a molecule through the plasma membrane can be used:

\[
M_m = P_m \frac{FV_m (C_m) \exp \left( \frac{FV_m}{RT} \right)}{RT \left( 1 - \exp \left( \frac{FV_m}{RT} \right) \right)}
\]

and

\[
P_m = \frac{M_m}{\left( \frac{FV_m}{RT} \right) \left( C_m \exp \left( \frac{FV_m}{RT} \right) \right) / \left( 1 - \exp \left( \frac{FV_m}{RT} \right) \right)}.
\]

where \( F, R, \) and \( T \) have their usual significance, \( P_m = \) plasma membrane permeability, \( M_m = \) efflux of dye across the plasma membrane, \( V_m = -80 \) mV, and \( C_m = \) the concentration of dye in the injected cell.

### TABLE I

**NEKUS SURFACE AREA**

| Septal membrane area | Nexal particles/\( \mu \text{m}^2 \) |
|----------------------|---------------------|
| 9.65 \( \times \) 10^2 \( \pm \) 5.6 \( \times \) 10^2 \( \mu \text{m}^2 \) \( (n=19) \) | 292 \( \pm \) 210 \( (n=16) \) |
| Average total Nexal surface area | Average total channel area |
| 4.32 \( \times \) 10^2 \( \mu \text{m}^2 \) | 8.8 \( \mu \text{m}^2 \) |

**Cell volume**

3.4 \( \pm \) 1.7 nl \( (n=19) \)

**Axon diameter**

65 \( \pm \) 17 \( \mu \text{m} \) \( (n=19) \)

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**Fluorescence Decrease with Light Exposure**

Experiments were performed in an attempt to measure the amount of decrease in fluorescence in time with exposure of the dyes to light. Tubes were drawn out to 40-\( \mu \text{m} \) diameters with 1-cm lengths. They were filled with 0.5 mM dye and the tube ends sealed. The tubes were photographed with 40-s exposures at varying time intervals. Densitometric scans were made of each exposure, and the area under the curve was calculated.

With such photographic exposure no more than 1% of the total area of densitometric scans was lost with any of the dyes. The interval of time in total darkness between exposures was independent of fluorescence loss. The longer the light exposure, the greater the loss.

**Dye Binding**

A Bio-Rad dialysis apparatus (Bio-Rad Laboratories, Richmond, Calif.) was used to determine the extent of cytoplasmic binding of each dye. A 5-ml sample of 0.2 M KCl and 40 \( \mu \text{M} \) of dye with no cytoplasmic protein was dialyzed against 100 ml of 0.2 M KCl and equilibrium was attained within 3 h for all the dyes. Earthworm nerve cords were homogenized and spun at 40,000 g for 30 min. The 5 ml of supernate containing 1 mg/ml protein (determined by the Lowry method) and a dye concentration of 40 \( \mu \text{M} \) was dialyzed against 100 ml of 0.2 M KCl for 3 h. Any difference in the absorption between the sample and the 100-ml vol after computation of the dilution factor (21:1) was taken to be caused by binding. If the average molecular weight of proteins was assumed to be 1 \( \times \) 10^4-1.5 \( \times \) 10^4 then the ratio of protein to dye was 2:1. Table II gives a summary of
binding for all the dyes. The binding values are estimates, and it should be noted that
the homogenates may not behave at all in binding properties like a living neuron.

RESULTS

No alteration in the action potential or resting potential could be observed
before or after iontophoresis of any of the seven dyes. When preparations were
impaled, if the resting potentials were $<-60$ mV and action potential height
$<80$ mV, the preparations were discarded.

Morphological studies of the septa were initiated by Stough (1930), and by
using light microscopy, he showed that the septa traverses the axon with
relatively little folding. Gunther (1975) reconfirmed the simple shape of the
septum with no folding using electron microscopy. Investigation of the septa by
Hama (1959) showed the membranes to be closely opposed, and vesicles were
shown in close association with the septal membranes. Dewey and Barr (1964)
showed the septa contained nexuses. Fig. 3 shows electron micrographs of

| TABLE II |
| INTRACELLULAR DYE CONCENTRATIONS AND CYTOPLASMIC BINDING |

| Dye                        | Initial dye concentration | Cytoplasmic binding |
|----------------------------|---------------------------|---------------------|
| Fluorescein                | 0.41±0.2 (n=4)            | 6                   |
| Dichlorofluorescein        | 0.44±0.28 (n=5)           | 3                   |
| Dibromofluorescein         | 0.50±0.3 (n=3)            | $>1$                |
| Diiodofluorescein          | 0.12±0.01 (n=3)           | 2                   |
| Tetrabromofluorescein      | 0.17±0.08 (n=5)           | $>1$                |
| Aminofluorescein           | 0.20±0.15 (n=2)           | 50                  |

freeze-fractured median giant axon septa. Particles ranging in size from 11 to 15
nm in diameter are in the P face (Branton et al., 1975) with pits in the E face.

Fig. 3 shows three different appearances for septal membranes. In Fig. 3a
there is some folding of the septum but this is seen rarely. The nexal particles
are loosely aggregated and are numerous over the surface of the septal
membrane. In many cases the septal membranes have only a few nexal particle
aggregates (Fig. 3b). Anywhere along the septum, vesicles like those seen in Fig.
3b may be seen (Hama, 1959). The vesicles are the same size as those seen in thin
section (Hama, 1959). A third case is typically seen in the septum (Fig. 3c) where
the particles and pits are not aggregated but are separated. The spacing varies
but is usually about 26 nm. Typically, the particles are found in the P face and
pits are in the E face. The septum has an unusual number of particles which
appear on the E face (Fig. 3c).

Fig. 4 illustrates two densitometric tracings made 1 and 15 min after termina-
tion of iontophoresis of the dye, dichlorofluorescein. Photographs of the
negatives used to make the microdensitometric tracings are also included in Fig.
4. Note the shift in the curve with time indicating that dichlorofluorescein
diffused beyond both septa. All the other dyes used showed similar shifts but
the amount of dye that diffused through the septa for a given time interval
diminished with increasing molecular weight.
FIGURE 3. (a) Freeze-fracture of earthworm median giant axon septum. Particles (connexions) are found predominantly in the P face and are loosely aggregated. Magnification × 29,620. (b) Freeze-fracture of septum showing a loosely aggregated nexus but an otherwise junction-free surface. Note also the vesicles in the cytoplasm adjacent to the septum. The vesicles varied in size from 95.0 to 40.0 nm. Magnification × 37,000. (c) Freeze-fractured septum where the pits and particles are spaced. The membrane surface is relatively flat. There are a large number of particles which appear in the E face. Magnification × 43,900. Arrows in lower right corner indicate shadow direction.
Table I shows data from 16 freeze-fractured septa. The total area of the septa is given as well as the average particle density (292 particles/μm²). Each particle averages 13.5 nm in diameter and from this the total surface area of nexus was determined. Both Matter (1973) and Spira (1971), using thin-sectioned material, showed that intercalated disks of mammalian myocardium contained 10-6% nexus, respectively, which is similar to the total nexus surface area of the septum. It has been proposed that the nexus particles contain a channel (2.0 nm in diameter; Weingart, 1974). Each conducting channel is presumably surrounded by insulation material. Using the total surface area of the channels to calculate permeability gives high values because the total surface of channels cannot account for frictional forces and charges that may be present in each channel which would tend to increase the resistivity of each channel. In addition, access to the total surface area would be much easier than to any one 1.5- to 2.0-nm channel of many million present. Table I also gives values for total nexus surface area and total “channel” area. These areas were computed using the mean particle density of 292 particles/μm².

The loss of total area under the curves from one time to another may be considered the result of the loss of dye which diffuses through the plasma membrane or inactivation of dye molecules due to previous light exposure (quenching) or a combination of both events. If it is assumed that for every photographic exposure 1% of total area is lost as a result of quenching and that any other loss is owing to efflux of the dyes across the plasma membrane, then a plasma membrane permeability can be computed for each dye. The computation is the same as that for septal membrane permeability using the cell surface area minus the septal membrane nexusal surface area. Inasmuch as the dyes are
negatively charged and the dyes are moving through the plasma membrane ($V_m = -80$ mV), Eq. 3 was used to compute plasma membrane permeability. The ratio of nexal membrane permeability to plasma membrane permeability is given in Table III. For both aminofluorescein and tetraiodofluorescein <1% of the area was lost, making accurate calculations of plasma membrane permeability impossible. Tetraiodofluorescein was difficult to iontophorese into the axon in concentrations large enough to record fluorescence. In those cases where fluorescence could be observed, no dye could be observed traversing the septa.

Aminofluorescein had a septal membrane permeability of $5.1 \times 10^{-7}$ cm/s, taking into account the binding. If no binding occurred, the permeability would be $2.5 \times 10^{-7}$ cm/s. During and after aminofluorescein iontophoresis, the action potential and resting potential showed no significant change, but after 2 h some depolarization was observed.

A summary of septal membrane permeability and flux to the various dyes across the septal membranes is given in Table III. Note that both permeability and flux decrease with increasing molecular weight. Permeability of septal
membranes to the six probes is graphed against molecular weight in Fig. 5. The correlation coefficient for the line drawn through the points is −0.95.

Examples of microdensitometric scans of a fluorescein injection are shown in Fig. 6. The time intervals were 4, 50, and 90 min. From tracings like those of Fig. 6, permeability at varying time intervals was calculated and permeability remained constant for any of the dyes used. The densitometric tracings indicate that dye moved across the septa and diffused through the axoplasm. It can be seen from the tracings that the system moved toward equilibrium. The nexal membrane permeability calculated from Fig. 6 for the 50-min time interval was $6.0 \times 10^{-6}$ cm/s and $5.7 \times 10^{-6}$ cm/s for the 90-min interval. In Fig. 5 the total
area decreased by 5.5% with the 50-min interval and 9% with the 90-min interval. Taking quenching into account (1% loss with each exposure), the total dye movement across the plasma membrane was 4.5% for 50 min and 7% for 90 min. There should be a lesser loss of dye across the plasma membrane between the 50- and 90 min interval because the dye concentration had decreased in the injected cell. The calculated plasma membrane permeability for the 50-min interval was $8.9 \times 10^{-9}$ cm/s and for the 90-min interval it was $8.1 \times 10^{-9}$ cm/s with a calculated initial dye concentration of 0.4 mM fluorescein and a cell volume of 3.0 nl. The profiles of the injected cell showed considerable variation in density. The fact that the axon at normal body length is folded in an accordion-like fashion may explain an otherwise unexplained variation in the densitometric scan.

**Correlation of Data with Passive Diffusion**

A diffusion model system such as two cylinders filled with solutions of similar composition and an interfacing resistance is equivalent to adjacent axoplasm interfaced by the nexal membrane of the septum. Diffusion in this system can be described by:
where \( D \) is the diffusion coefficient \( (\text{cm}^2/\text{s}) \) and \( C \) is the concentration. The interface or septum is located at \( x = 0 \).

It is assumed that the concentration of the diffusing solute in the region \( x > 0 \) is uniform \( (C_0) \) at \( t = 0 \) and for \( x < 0 \) the concentration is zero for \( t = 0 \). \( C_1 \) will denote the concentration for \( x > 0 \). \( C_2 \) will denote the concentration for \( x < 0 \).

\[
\frac{\partial^2 C}{\partial x^2} = \frac{\partial C}{\partial t},
\]

Figure 6. Fluorescein was iontophoresed into an axon for 15 min. Photographs were taken 4, 50, and 90 min after the iontophoretic period ended. Densitometric tracings of the three negatives were made. The line drawn at the base of each trace served as a base line for area and amplitude calculations. Note that the tracings indicate dye movement into adjacent cells. The decrease in density (therefore concentration) is roughly exponential with distance along the axon. The horizontal bar under the first tracing represents a distance of 200 \( \mu \text{m} \). Permeabilities calculated for the various time intervals were the same.

The two boundary conditions are (a) \( D_1 \frac{\partial C_1}{\partial x} = D_2 \frac{\partial C_2}{\partial x} \) at \( x = 0 \), and (b) \( D_1 \frac{\partial C_1}{\partial x} = P_n (C_2 - C_1) = 0 \) at \( x = 0 \) where \( P_n \) = permeability \( (\text{cm/s}) \) across the interphase. \( D_1 \) is the diffusion coefficient for \( x > 0 \) and \( D_2 \) is the diffusion coefficient for \( x > 0 \) and \( D_2 = D_1 \). The nexal membrane permeability of the interphase equals \( P_n \). Integration leads to solutions for \( C_1 \) and \( C_2 \) as a function of distance and time which are given by Crank (1956, p. 40). The cylinders with an interphase at \( x = 0 \) are assumed to go on to plus and minus infinity whereas the distance from septum to septum is usually 1–2 mm (Brink and Barr, 1977). Therefore short time intervals after injection were used to allow comparison of experimental
results with the diffusion model system because short time intervals would allow
the best possible approximation of the boundary conditions.

Two examples of comparisons between experimental and theoretical results
are given in Fig. 7. Fig. 7A shows (left-hand side) a densitometric trace from
negatives made of a dichlorofluorescein injection of the axon. The photographs
were made 30 s and 3 min after termination of injection period. The permeabil-
ity computed from the densitometric tracings was $2.1 \times 10^{-5}$ cm/s. Fig. 7A
(right-hand side) also contains curves of $C_1$ and $C_2$ gotten from the Crank
formalization made at $t = 30$ s and 3 min where the interphase permeability was
$2 \times 10^{-5}$ cm/s and the diffusion coefficient was $5 \times 10^{-7}$ cm$^2$/s. At $t = 0$, $C_1 = 1$
and $C_2 = 0$. Fig. 7B shows densitometric traces of negatives taken 1 and 16 min
after termination of the iontophoresis of dibromofluorescein. The permeability
calculated from these tracings was $1 \times 10^{-5}$ cm/s. Tracings of $C_1$ and $C_2$ are
shown at 1 and 15 min where the interphase permeability was $7 \times 10^{-6}$ and the
diffusion coefficient was $9 \times 10^{-6}$. Once again at $t = 0$, $C_1 = 1$ and $C_2 = 0$. These
examples are used to show a correlation between the densitometric data and the
diffusion model. From the comparison it is apparent that the dyes are moving
through the septa via passive diffusion processes. One important difference is
the behavior of the injected cell region of the densitometric tracing and that of
the $C_1$ region. This disparity has as yet no full explanation.

In all cases the permeabilities calculated from densitometric tracings when
used in the Crank (1956) equations gave tracings of $C_2$ that were in good
agreement with the densitometric tracings. The diffusion coefficients for
fluorescein was $1 \times 10^{-6}$ cm$^2$/s, $5 \times 10^{-8}$ cm$^2$/s for diiodofluorescein, and $1 \times$
$10^{-4}$ cm$^2$/s for tetrabromofluorescein. In each case, short time intervals of $\leq 16$
min were used. In each instance only one densitometric trace per dye was
analyzed with respect to the Crank equations. Fig. 8 is a comparison of nexal
membrane permeability of various probes (Weidmann, 1966; Weingart, 1974;
Pollack, 1976), including those of this study. Permeability is plotted against
molecular weight of the probes. In all these cases the total nexus surface area is
used in computation of flux data from which permeability is computed.

DISCUSSION

The molecular weight of the fluorescent probes used here varied from 333 to
652, and the nexal membrane permeability varied from $5.4 \times 10^{-6}$ to $4.0 \times 10^{-6}$
cm/s using the nexus surface area as the conduction proportion of the septal
membrane.

Calculation of the potassium permeability can be made from the resistance
data (Brink and Barr, 1977) and use of the equation:

$$G_K = K_1 \times P_K \times (F^2/RT) \quad (\text{Weidmann, } 1966)$$

where $G_K =$ conductance of the disk for potassium ions, $P_K =$ permeability of
disk to potassium, $F$, $R$, and $T$ have their usual significance.

$K_1 =$ potassium concentration in the myoplasm. If the disk conductance and
permeability to potassium is assumed to be nexal membrane conductance and
permeability, then Eq. 5 can be rearranged so that
Figure 7. (A) Comparison of densitometric tracing made from negatives of a dichlorofluorescein injection and the Crank equations (1956). Photographs were made 30 s and 3 min after termination of dye injection. $D =$ diffusion coefficient and $P =$ permeability. The permeability computed from the densitometric tracings was $2.1 \times 10^{-5}$ cm/s. Densitometric data are displayed on the left side of the figure, curves from the Crank formalism are shown on the right. On the right, values of $x$ from 0 to negative values of $x$ represent the adjacent cell whereas positive values of $x$ represent the injected region. (B) A similar comparison to A only the dye used was dibromofluorescein. The permeability computed from densitometric tracings was $1 \times 10^{-5}$ cm/s (left side).
Figure 8. Semi-log plot of permeability vs. molecular weight. Triangles are values taken from Weingart (1974). The open circle is a value for fluorescein taken from Pollack (1976). Filled circles are data present in Fig. 5. The data for aminofluorescein are also shown. Molecules summarized by Weingart (1974) were K⁺, tetraethylammonium (TEA), and Procion Yellow.

\[ P_K = \frac{G_K}{K_1 \times (F^2/RT)} \]  

(6)

where \( P_K \) is the nexal membrane permeability to potassium.

Brink and Barr (1974, 1977) calculated a nexal membrane resistance of 6 ohm cm² in the septa of the median giant axon of earthworm. Using the surface area data presented here, the resistance of the nexal membrane drops to 0.3 ohm cm² for the nexal membranes of the septa. If it is assumed that the internal
potassium ion concentration is $150 \times 10^{-6} \text{ mol/cm}^3$, then the $P_K$ for the septal nexus can be computed from Eq. 6 to be $5.0 \times 10^{-3} \text{ cm/s}$.

McNutt and Weinstein (1973) and Matter (1973) from morphological studies estimated the nexal membrane pore to be between 1.0 and 1.5 nm in diameter. Radioactive $K^+$, TEA, and Procion Yellow were able to traverse a nexal membrane, indicating that the pore was approximately 1.0 nm. In the developing squid the dye Chicago Blue (1,000 mol wt) could traverse nexal membranes (Potter et al., 1966). The molecular dimensions of Chicago Blue are not known. Models of fluorescein, dichlorofluorescein, dibromofluorescein, diiodofluorescein, tetrabromofluorescein, and aminofluorescein were made using conventional bond angles and lengths. The probes vary in their widest dimension from 0.9 to 1.5 nm. Inasmuch as all the probes except aminofluorescein are negatively charged at physiological pH, they are assumed not to be hydrated. The largest diameter is 1.3 nm, therefore the pore must be at least 1.3 nm in diameter to accommodate such molecules (tetrabromofluorescein). The largest dimension of tetraiodofluorescein is 1.4 nm.

The comparison of the densitometric tracings with solutions of a diffusion model given by Crank (1956) are good with respect to the adjacent cell region and the solution of $C_2$. The permeabilities used for the model are the same or similar in magnitude to those computed from the densitometric tracings. However, there is not as good a correspondence between densitometric tracings of injected cell regions and $C_1$ with varied time. Possible explanations for this may be some change of the diffusion coefficient in the injected cell region due to high dye concentration, or possibly some form of axoplasmic flow not seen with low concentrations of dye in adjacent segments. There may be many other explanations for this presently unexplainable derivation from theory.

Estimates of cellular dye concentration were undertaken to determine whether the amount of dye injected was changing the osmotic conditions of the cell significantly and to allow an estimate of the concentration gradient across the nexus. Figs. 1 and 2 were used as standard curves to estimate dye concentration in the cytoplasm. There was no case where the amplitude of a densitometric trace exceeds that of Fig. 1. Variations in Fig. 1 may be the result of wall thickness differences.

If the permeabilities are calculated using just the "channel" surface area, then the permeabilities are raised by a factor of approximately 50. Therefore, the fluorescein permeability would be $2.7 \times 10^{-3} \text{ cm/s}$. Use of values this high in the Crank (1956) model bring the model to equilibrium within 10-20 min and the diffusion across the interphase greatly exceeds that of the densitometric tracings. Assuming that the permeability of fluorescein to the septa which best fits the Crank equations is correct ($5.4 \times 10^{-3} \text{ cm/s}$) and that each particle has a 1.5- to 2.0-nm channel, then the channels have a factor of 50 greater resistance to diffusion than axoplasm.

Another point is the use of short time intervals for analysis of computed data and the Crank equations. With short time intervals, used just after injection termination, good agreement for permeability values was possible. If longer intervals were used (30-50 min), the permeability value used in the Crank model...
was always less than the computed value by a factor of 2–5. This error is most likely due to the fact that at longer time intervals the experimental computed values are gotten under conditions where the boundary conditions are not met for the model. Fig. 5 shows an apparent linear relationship for permeability vs. molecular weight, and this may be due to the fact that surface charge density of the molecules is different. Plotting the points on a logarithmic plot shows that they all fall in the range of other probes used on nexal membranes (Fig. 8).

The permeability of aminofluorescein to nexal membranes is much lower than the permeability of any of the anion dyes. The reasons for this may be that aminofluorescein binds to nexal membranes as it does to cytoplasmic proteins or that the net charge of aminofluorescein is different from those of fluorescein, dichlorofluorescein, dibromofluorescein, diiodofluorescein, and tetrabromofluorescein. Fluorescein and the fluorescein halogen derivatives would have a net negative charge at physiological pH whereas aminofluorescein would have a net positive charge or be neutral.

The nexal membrane permeability in the septum of the median giant axon of earthworm ranges from $5.4 \times 10^{-5}$–$4.0 \times 10^{-6}$ cm/s for mol wt of 333–652. The three-dimensional structure of the individual dyes will determine their ability to diffuse across the nexus, but the data given here would suggest that molecules in the range of 1,000 mol wt represent the upper limit for molecules to diffuse across a nexal membrane.

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