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SOLVENT-SOLUTE INTERACTIONS WITHIN THE NEXAL MEMBRANE

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The nexus, or gap junction, is thought to contain aqueous intercellular channels with diameters of ~1.5 nm (2, 13). Both physiological (20, 21) and anatomical data (14) have contributed to the aqueous channel concept. More recent findings in the septate giant axon system (7) have shown that the temperature dependence of junctional permeability to dichlorofluorescein decreases sharply (blocks) at 4° ± 0.5°C in H_2O and 6°C in D_2O. Action potential propagation still continues across the junction well below 4°C in either solvent. The block temperature can be defined as that temperature at which no appreciable dye diffusion can be observed across the junction for one to two hours. In reality the permeability of the junction has fallen below the ability of the photomultiplier system to perceive the movement of dye across the septa over the time interval indicated. Although the diffusion rates were unmeasurable at block temperature, some dye transfer was observable with long time intervals (14–20 h). The junctions studied were nexuses contained within the septa of the septate median giant axon of earthworm (5, 8, 10).

To elucidate further the nature of ion and anion movement within the junction, the involvement of solvent-solute, solute-channel and solvent-channel interactions was investigated for a number of fluorescent probes in both deuterium oxide (D_2O) and water (H_2O). The probes used were carboxyfluorescein, Lucifer Yellow CH, Lucifer Yellow 37 and diiodofluorescein (Fig. 2). In addition, junctional membrane conductance was monitored in D_2O and H_2O (4). Temperature regulation was accomplished by use of a Cambion temperature control device. The pH and pD were adjusted such that both equaled 7.3 (19).

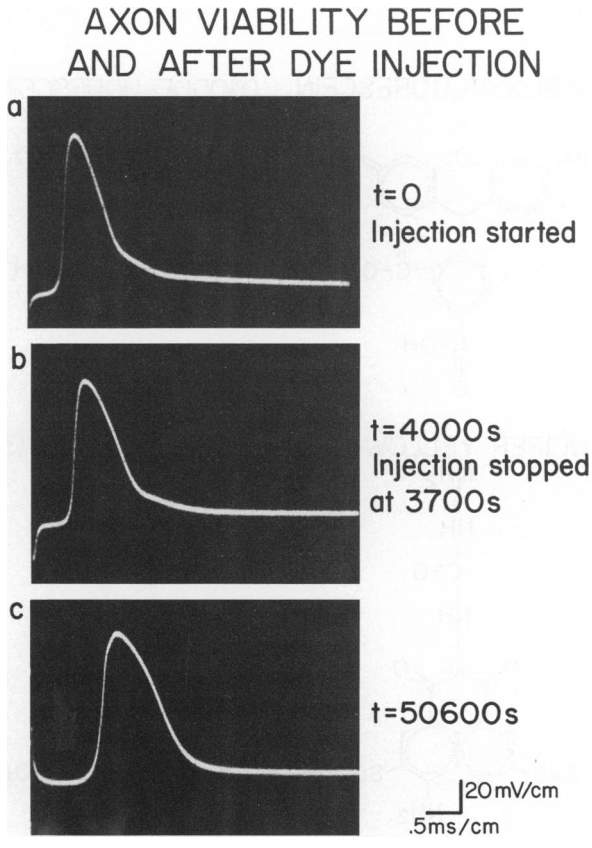

FIGURE 1 A microelectrode filled with dye (Lucifer Yellow CH) was inserted into an axon in H_2O saline. *a*, an action potential is shown. Stimulating electrodes were 1 cm away from the site of recording. *b*, the same cell 4,000 s later after a 3,700 s injection. The microelectrode was removed. The cell was scanned and diffusion monitored and *c* at 50,600 s a 3 M KCL microelectrode was inserted into the same cell and the nerve cord was once again stimulated. The threshold was larger and the cell was partially depolarized (≈10 mV).

RESULTS AND DISCUSSION

The fluorescent probes were inserted by iontophoresis into the septate axon system. Hyperpolarizing current pulses 50

nA of 100 ms duration were applied every 150 ms to fill cells. Cell viability was assessed by monitoring resting and action potential amplitudes before and after dye iontophoresis. In Fig. 1 an action potential is shown at the beginning of an injection (1 *a*), at the termination of the injection (1 *b*) and 14 h later (1 *c*). Experiment duration and dye exposure did cause some membrane depolarization, ~10 mv after 12–14 h. The diffusion of dye above the block temperature in D_2O or H_2O is easily observed (Fig. 3) at temperatures of 6.9°C and 10°C. Changes in dye concentration along the septate axon were observed by fluorometry (7). Fluorometric scans along the long axis of the axons were made at a fixed excitation wavelength. The photomultiplier was also set to respond to the appropriate fluorescent wavelength. The distribution of the probe within a cell and within adjacent cells could then be ascertained as shown in Fig. 3 *a*. The example given here shows Lucifer Yellow CH in H_2O at various time intervals and temperatures. Note the lack of diffusion across the septa at temperatures below 6.9°C, while above this temperature diffusion into the connected cells is obvious. Experiments like that of Fig. 3 *a* were done on all probes in

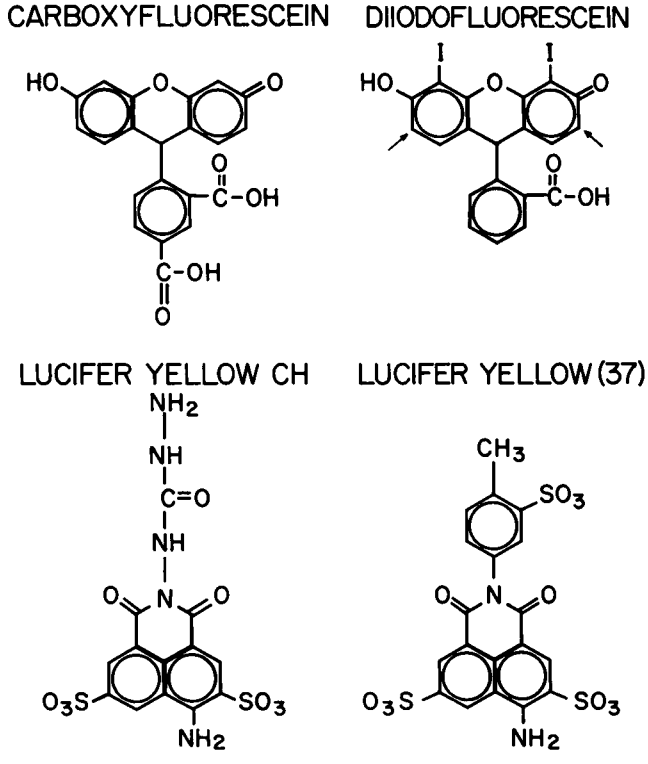

FIGURE 2 Diagrammatic representation of the fluorescent probes used in this study. 2, 7 dichlorofluorescein is not shown. The chloro groups are placed on the positions marked with the arrows. 4, 5 dichlorofluorescein was not used. Note that Lucifer Yellow (CH) has two sulfate groups with p*K*s of 0.7 (Stewart 1978). Lucifer Yellow (37) contains three sulfate groups. The Lucifer probes were obtained from W. Stewart. Carboxyfluorescein has two carboxy groups while diiodo and dichlorofluorescein have one each. The ionized portions of the probes are those regions most responsible for solvent-solute interaction resulting in hydration shells.

H_2O and D_2O. For comparison, Fig. 3 *b* shows the relationship between septal (nexus) membrane conductance and temperature in both solvents. There is no slope change or step change in the conductance as there are for the majority of the dyes. The Q_{10} for junctional conductance was 1.4 in H_2O and ~1.55 in D_2O. These data indicate that the ions responsible for current flow in the junctions experience an environment much like that of bulk solution. Assuming that ions and dye molecules utilize the same diffusion pathway, the large difference in size between these two kinds of probes may account for the disparity between the conductance and permeability data.

Table I lists the temperature at which a particular probe experiences a pronounced decline in its permeability (block) in H_2O and D_2O. Over the time intervals used (2 h) the dye diffusion appeared to be completely blocked.

Arnett and McKelvey (1), Heppolette and Robertson (9), and Bockris and Reddy (3) have described the solvent structure of H_2O and D_2O. The increased viscosity of H_2O at low temperature is believed to be the result of increased order in its structure that impedes the motion of a solute as it moves through solution. The stronger intermolecular forces of heavy water exaggerate these effects (16). Charge interactions cause ionized solutes to be enveloped by a solvent sheath. If the solute and its hydration shell can be considered as a single diffusing entity, then D_2O would be expected to impede solute mobility further by creating at any one temperature a larger hydration shell than H_2O.

Solvent-solvent, solvent-solute and solvent-channel interactions provide an excellent basis for interpretation of the results given in Table I, but temperature effects on channel diameter must also be considered. If the intercellular channels undergo a conformational change with cooling, such as reduced diameter, then the dyes (0.9–1.0 nm) are eliminated from the diffusion pathway. Therefore, the same "block" temperature might be expected for all the probes. The permeability data for H_2O in Table I certainly suggest this possibility (4°–6°C). The conductance data can be explained on the basis of the small size of K^+. The only dye which does not fit this explanation is diiodofluorescein. Its diffusion rate appears to be unaltered at 4°C in either solvent. It is a weak acid with a p*K* around five (12, 17). The D_2O data make necessary another explanation. It is not channel alteration but solvent effects that are responsible for dye impermeability of the junction. Each probe displays a different temperature block in D_2O easily distinguishable for the others, unlike the H_2O data. The Lucifer compounds have p*K*'s of 0.7 (17) and carboxyfluorescein has p*K*'s in the range of three to four. The dyes have varied numbers of ionized groups. Recall from Fig. 2 that Lucifer Yellow 37 has three SO_3 groups, Lucifer Yellow CH has two, carboxyfluorescein has two carboxyl groups and dichloro- and diiodofluorescein have one carboxy group. The solutes with the greatest number of ionized groups would have a greater number of attending water molecules than solutes with a lesser number of

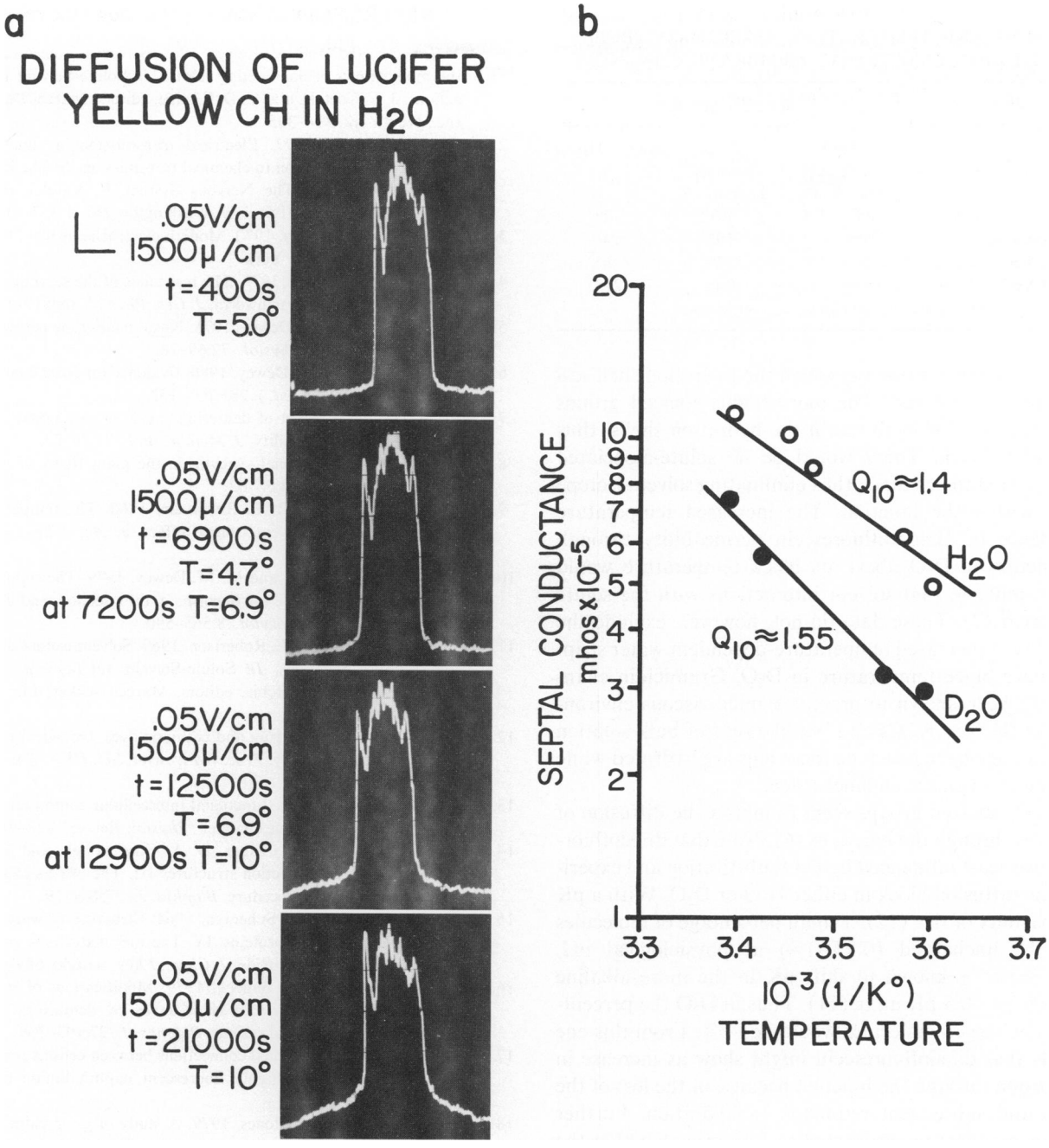

FIGURE 3 *a*, an axon was injected with Lucifer Yellow (CH) in H_2O saline and the distribution of dye was followed for 21,000 s at various temperatures. The concentration of the dye in the cells is proportional to the area under the curves. At the termination of the injection the preparation was cooled to 5.0° ± 0.5°C and held at that temperature for 7,200 s. At that time the temperature was raised to 6.9°C. The preparation was held at 6.9°C for an additional 5,200 s (12,900 s). The temperature was finally set to 10°C for another 8,100 s (21,000 s). Diffusion across the junction is seen at 6.9°C and above. *b*, septal membrane conductance was measured in both solvents at different temperatures. The data is plotted as an Arrhenius plot. Note that there is no block or slope change in the relationship (conductance vs. temperature) in either solvent. The method used to measure conductance was that of Brink and Barr (1977).

groups. The charge groups with the most dense electrical field are also thought to maintain the largest hydration shell(s) (3). Cooling in either solvent is thought to cause a further increase in hydration shell size. The temperature block of the dye is the temperature where little or no transjunctional diffusion is possible because of the size of the solute-solvent complex. The temperature block can be interpreted as that temperature at which most hydrated (deutrated) dye molecules are too large to diffusion through the junction.

The results may also be explained on the basis of water stripping. Each temperature at which a diffusion "block"

TABLE I
SOLVENT AND TEMPERATURE EFFECTS ON PROBE DIFFUSION IN JUNCTIONAL MEMBRANE CHANNELS

Probe	Block Temperature		
	H_2O	D_2O	$D_2O - H_2O$
Diiodofluorescein	no block down to 4°C	no block down to 4°C	—
Dichlorofluorescein	4°C ± 0.5	6°C	2°
Carboxyfluorescein	5°	19°	14°
Lucifer Yellow CH	6°	12°	6°
Lucifer Yellow 37	6°	28°	22°
Junctional Conductance	no block	no block	—

occurs is the temperature at which the hydration shell can no longer be removed. The more highly ionized groups have a greater ability to retain the hydration shells, thus effecting a block. There would be no solute-associated solvent within the channel, thus eliminating solvent isotope effects within the junction. The increased temperature dependence of dichlorofluorescein permeability through the junction in D_2O above its block temperature would seem to indicate that solvent interactions with the solute are present (7). These data do not, however, exclude the possibility of increased temperature-dependent water stripping above block temperature in D_2O. Gramicidin channels have been shown to present a microviscous environment for the ions K, Cs and Na like that of bulk solution (18), leaving one to conclude these ions are hydrated while diffusing through the channel space.

Finally, ionized groups seem to inhibit the diffusion of molecules through the junctions (6). Note that diiodofluorescein was least influenced by D_2O substitution and experienced no diffusion block in either H_2O or D_2O. With a pK in the vicinity of five (12), a small percentage of molecules would be uncharged (0.5%–1%) at physiological pH. Heavy water is known to shift pK in the more alkaline direction by ~0.5 pK units (11). Thus in D_2O the percentage of uncharged molecules would be ~5%. From this one predicts that diiodofluorescein might show as increase in its diffusion through the junction because of the loss of the charge and subsequent reduction in hydration. Further experiments must be performed to determine whether the uncharged form of a probe is the predominant diffusing species.

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