curred with the following frequencies: eight 0.25-hour exposures (= 2 hours); eight 0.50-hour (= 4 hours); and two 1.00-hour (= 2 hours), yielding a total of 8 hours of illumination. The dark periods were represented as follows: three 0.50-hour exposures (1.50 hours); five 0.75-hour (= 3.75 hours); eight 1.00-hour (= 8.00 hours); one 1.25-hour (= 1.25 hours); and one 1.50-hour (= 1.50 hours), affording a total of 16 hours of darkness.

No.	Duration	
	Light period (hr)	Dark period (hr)
1	0.25	1.00
2	0.25	1.00
3	0.50	0.50
4	0.25	0.75
5	1.00	0.50
6	0.25	0.75
7	0.25	1.00
8	0.25	1.00
9	0.50	0.75
10	0.50	1.00
11	0.50	1.00
12	0.25	1.00
13	0.50	1.50
14	0.25	1.25
15	0.50	0.75
16	0.50	0.50
17	1.00	1.00
18	0.50	0.75

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Toad Urinary Bladder: Intercellular Spaces

Abstract. *Vasopressin causes dilation of the intercellular spaces of the mucosal epithelium in toad bladder, an effect previously thought to result from enhanced net transepithelial water transport. Under conditions of zero net fluid transport, vasopressin exerted the same effect in seven tissues, which indicates that the width of the intercellular spaces cannot be taken as a reliable index of net transepithelial fluid transport.*

Under a variety of conditions, the size of the intercellular spaces in rabbit gallbladder epithelium depends upon the rate of fluid transport (*1*). In toad urinary bladder, the intercellular spaces are also enlarged after vasopressin is administered under the condition of a dilute mucosal medium (*2–4*); this effect has been thought to arise from increased net fluid transport (*2*). It has been observed, however, that application of vasopressin in the absence of an osmotic gradient, when no net transfer of water occurs (*5*), results in an enlarged or open configuration of the intercellular spaces (*4*). This finding suggests that factors other than rate of water flow are involved in the regulation of size of the intercellular spaces.

Urinary hemibladders were excised from doubly pithed toads; they were rinsed in Ringer solution and mounted in Lucite double chambers (*6*) so that there were adjoining experimental and control halves for each preparation. Short-circuit current was monitored in experiments with Na^+ Ringer solution (Na^+, 113.4; K^+, 3.5; Ca^{2+}, 0.9; Cl^-, 116.3; HCO_3^-, 2.4 m*M*; *p*H 7.4 to 8.2; tonicity, 216 to 226 milliosmoles per kilogram of water). Transepithelial electrical potential was followed in experiments with choline$^+$ Ringer solution (quantitative replacement of Na^+ with choline$^+$; 2 m*M* phosphate rather than bicarbonate). In three of these experiments, net water flow was measured with a volumetric technique (*6*) so that no net transfer occurred under the isotonic conditions imposed.

After the physiologic measurements, the tissues were fixed with glutaraldehyde and prepared (*7*) for examination with a Philips EM-200 electron microscope. To provide objective in-

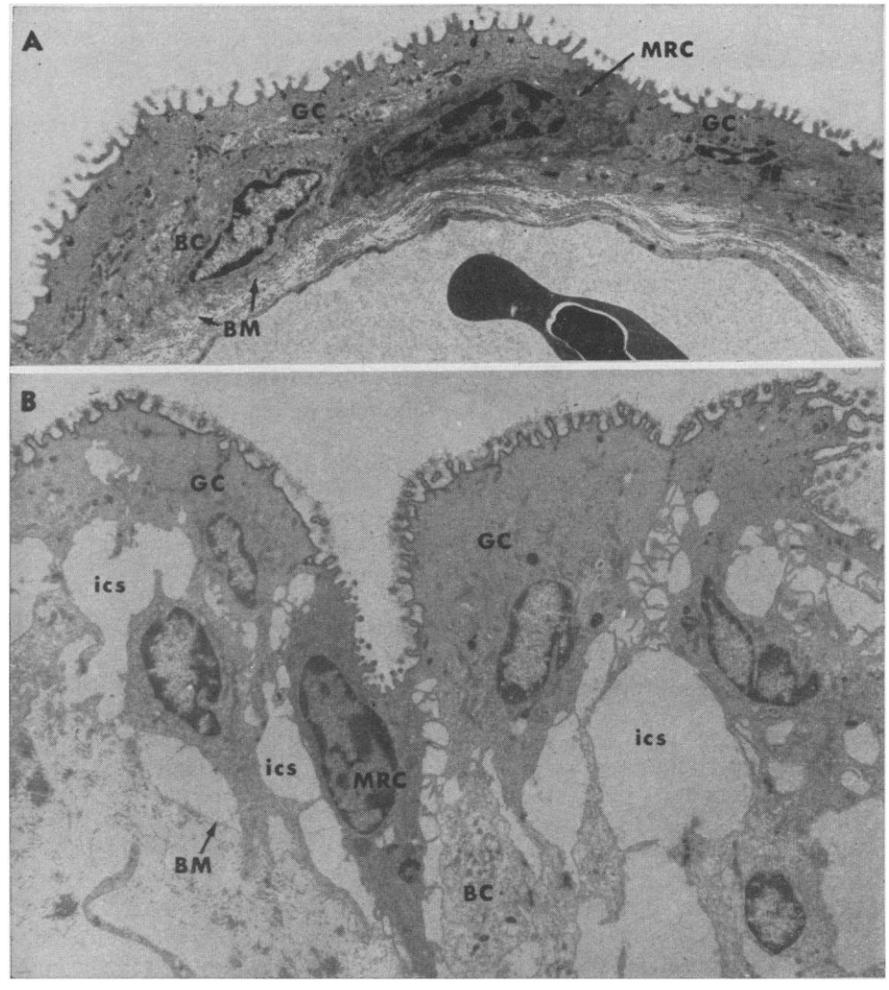

Fig. 1. Electron micrographs of toad bladder epithelium showing samples from the control and experimental portions of the same preparation. (A) Control tissue. Tissue was bathed on both surfaces with isotonic Na^+ Ringer solution; no vasopressin was added. At this magnification, there is no visible separation between neighboring cells or between cells and the basement membrane (*BM*); *GC*, granular cells; *MRC*, mitochondria-rich cells; *BC*, basal cells (× 4000). (B) Experimental tissue. Tissue was bathed on both surfaces with isotonic Na^+ Ringer solution. Vasopressin was added to the serosal bath 15 minutes prior to fixation; short-circuit current increased but net transfer of water was not measurable. The intercellular spaces (*ics*) are now markedly enlarged making cell margins clearly visible at low power (× 4000).

terpretation, one of us (D.R.D.) examined the tissues without prior knowledge of which was the experimental side.

Four hemibladders were bathed on both surfaces by isotonic Na^+ Ringer solution. Vasopressin (Pitressin) was added to the serosal medium of the experimental half of each preparation to a final concentration of 40 to 200 milliunits per milliliter. An identical volume of Na^+ Ringer solution was added to the serosal medium of the control half. The short-circuit current of all the experimental sides increased after addition of hormone. The tissues were fixed 15 to 20 minutes after addition of hormone.

The control side was characterized by closely apposed lateral margins (Fig. 1A). The sample treated with vasopressin showed enlarged intercellular spaces (Fig. 1B).

Three experiments were carried out in identical fashion with isotonic choline$^+$ Ringer solution. In the absence of Na^+, the addition of vasopressin did not alter the transepithelial potential, which remained close to zero. However, electron micrographs revealed that the results were unchanged from those obtained with Na^+ Ringer solution. In every case, the intercellular spaces of the experimental side were considerably more open than those of the control.

Vasopressin may increase the intercellular spaces in the absence of net transfer of salt and water. This increase may be related to another effect of vasopressin, the relaxation of the smooth muscle of toad bladder (*8*). The distribution of the submucosal smooth muscle and collagen may be such that muscle contraction results in increased pressure against the basement membrane, directed from serosa to mucosa. Since the basement membrane is porous (*9*), intercellular fluid would then be extruded into the submucosa, and the cells would be more closely apposed.

In other experiments (*10*), hemibladders were very loosely mounted, and one side was distended by an applied hydrostatic gradient. The nondistended control half revealed markedly open intercellular spaces, while the spaces of the distended half were tightly closed.

We conclude that although dilation of the intercellular spaces may result from increased net transepithelial water flow, other experimental conditions may produce similar morphologic changes. Therefore, dilation of the intercellular spaces in itself does not indicate increased net transport of water across epithelial membranes.

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Noncovalent Binding of a Spin-Labeled Inhibitor to Ribonuclease

Abstract. *The stable free radical 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl monophosphate has been synthesized; it binds to ribonuclease. The selective changes in the nuclear magnetic resonance spectrum of the enzyme produced by the free radical make it possible to define qualitatively the region of the enzyme to which it binds. The radical appears to occupy a site similar to that to which inorganic phosphate binds which is close to or within the active site of the enzyme.*

Stable free radicals, or spin labels, were first used for investigating the structure of proteins by McConnell and co-workers (*1*) and subsequently by others (*2–4*). Most of these investigators studied the electron spin resonance spectrum of a free radical covalently attached to a protein and obtained information about changes in the relative rotational freedom of the spin label and hence about changes in its environment on the protein under various conditions (*1, 2*). The unpaired electron of these free radicals also increases the relaxation rate of a nearby nucleus, and this effect can be used to give detailed information about the environment of the spin label. Sternlicht and Wheeler (*3*) showed that a marked broadening of the nuclear magnetic resonance (NMR) spectrum of lysozyme resulted in covalent attachment of a spin label to the protein, although specific effects were not observed. Mildvan and Weiner (*4*) have reported that the effects of a spin-labeled analog of nicotinamide-adenine dinucleotide (NAD) on the relaxation rates of the protons of ethanol and acetaldehyde in the presence of liver alcohol dehydrogenase (E.C.1.1.1.1) could be used to give detailed information on the mode of binding of substrates to the enzyme.

We have now studied the effects of a spin-labeled inhibitor on the NMR spectrum of bovine pancreatic ribonuclease A (E.C.2.7.7.16). The inhibitor used was 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl monophosphate (III). Ribonuclease is competitively inhibited by phosphate, sulfate, and arsenate (*5*), and by many phosphate monoesters (*6*). We hoped, therefore, that compound III would similarly bind to the active site of the enzyme.

Compound III was prepared as follows: A freshly prepared 2.0 molar solution of dimorpholinophosphobromidate (*7*) in chloroform (12.8 ml, 0.0255 mole) was added at room temperature to a chloroform solution (12.8 ml) of 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl (I) (*8*) (4.00 g, 0.0232 mole) and tribenzylamine (7.34 g, 0.0255 mole), which was left at room temperature in a sealed flask for 17 hours. More 2.0 molar reagent (6.4 ml) and tribenzylamine (3.67 g) were added, and the reaction mixture was set aside for another 24 hours. Chloroform was then removed at reduced pressure, and the residue was triturated with benzene (two 50-ml portions). Insoluble tribenzyl hydrobromide (10.09 g) was filtered off, the solvent was removed from the filtrate at 75°C (1 mm-Hg), and the residual dark red syrup (18.03 g) was chromatographed over silica gel (720 g) in chloroform containing increasing amounts of methanol (2 percent maximum). Tribenzylamine (5.53 g) was eluted first, and then 2,2,6,6-tetramethylpiperidin-1-oxyl-4-phosphoryldimorpholidate (II) (5.47 g) was eluted. Crystallization twice from cyclohexane containing a trace of ether