Ion and Water Transport in the Proximal Tubules of the Kidney of *Necturus maculosus*

GUILLERMO WHITTEMBURY

From the Biophysical Laboratory, Harvard Medical School, Boston

The nature of the transport of ions and water in the proximal tubule of the kidney has been a subject of extraordinary interest for a long time. In the mammalian kidney, 80 per cent of the fluid filtered in the glomerulus is absorbed by the proximal tubules. It was shown many years ago by Walker, Richards, and colleagues, for the amphibian (1) and mammalian kidneys (2), that under normal conditions, the fluid absorbed by the proximal tubule was isosmotic to the plasma, and that it was essentially of the same composition as the glomerular filtrate. Two possibilities were then considered to explain the mechanism of the absorption in the proximal tubule: (*a*) the primary step is an absorption of solutes, with water following passively, or (*b*) the colloid osmotic pressure exerted by the proteins circulating in the peritubular capillaries is sufficient to produce direct water absorption, the solutes following.

Wesson and Anslow (3) in experiments in dogs showed that in osmotic diuresis, Na and Cl were both absorbed against a chemical gradient and suggested the active nature of the Na transport; however, their conclusion was based on the assumption of a negligible effect of the distal tubule under their experimental conditions. That assumption has been subjected to criticism. They also did not consider electrical potential gradients.

The concepts of active transport have evolved from early suggestions of dependence of transport on metabolism. This, however, is not a sufficient definition. Two generally acceptable definitions of an active transport process will be considered. Rosenberg (4) has defined as active transport any transport which takes place against an electrochemical potential gradient. Ussing (5) has defined active transport as movement of a substance that cannot be explained by simple diffusion. This definition is more general than the one given by Rosenberg, but is difficult to apply in practice for it requires a knowledge of the rate at which the substance in question crosses the membrane by simple diffusion. Equation 1 (5) describes the flux of an ion moving independently across a membrane, in the absence of temperature gradient,

Rockefeller Foundation Fellow; on a leave of absence from Facultad de Medicina, Lima, Peru.
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hydrostatic pressure gradient, bulk flow of solution, other electrochemical potential gradients, and chemical reactions involving the species considered.

$$\frac{\Phi_{12}}{\Phi_{21}} = \frac{C_1}{C_2} e^{\frac{zF}{RT}(\psi_1 - \psi_2)} \quad (1)$$

In this equation, the assumption has been made that the activity coefficients are equal on both sides of the membrane. Φ_{12} and Φ_{21} are efflux and influx respectively, the subscripts 1 and 2 refer to inside and outside of the membrane, C is the concentration of the ion, z its charge, F the Faraday, Ψ the electrical potential, R the gas constant, T the absolute temperature.

The use of this formula requires the measurement of concentrations and fluxes of the species under study, and estimation of the transmembrane electrical potential difference in suitable experiments in which the other parameters mentioned are kept constant.

In the experiments to be reported below, the stopped flow microperfusion technique developed in this laboratory (6) permits the measurement of all the separate parameters in equation 1. This technique has been used to study nature of the transport of sodium, chloride, and water in the proximal tubule of the kidney *in situ*. Because of their size and distribution, the proximal tubules of the amphibian *Necturus maculosus* are the most convenient for such a study.

We shall present: first, a description of the method; second, evidence for the dependence of water and sodium fluxes on metabolically driven processes; third, evidence that sodium is transported against both a chemical and electrical gradient whereas chloride is not; fourth, evidence that the colloid osmotic pressure is not sufficient to account for the water fluxes actually observed; and finally, some considerations regarding electrical potential differences across the proximal tubules.

1. Stopped Flow Microperfusion Technique

The proximal tubule of the *Necturus* kidney has the following average dimensions (11): diameter 140 μ, length 14 mm., surface area 6.16×10^{-2} cm.^{2}, and a volume of 0.215 μl. Its anatomical configuration is shown in Fig. 1.

The stopped flow microperfusion technique involves the use of micropipettes, with tips of the order of 10 to 20 μ in diameter, connected with suitable holders to two micromanipulators. The work is performed under a dissecting microscope. The volume of the samples is of the order of 0.06 to 0.2 μl. Adequate precautions to avoid evaporation and contamination of the samples have to be observed. After exposure of the kidney, the glomerulus is punctured with a micropipette and blocked with mineral oil (Fig. 2). With another micropipette a perfusion fluid of known composition is intro-

duced into the proximal tubule. After 20 minutes the tubular fluid is collected from the end of the proximal tubule and analyzed. Changes in C^{14} inulin concentration are used as an index of water movement (6), since the tubular wall can be considered essentially impermeable to inulin. When Na fluxes were studied, total Na concentration was measured in the modified flame-photometer of Solomon and Caton (9); Na^{24} samples were counted in a

FIGURE 1. Scale drawing of a representative nephron of *Necturus* taken from Kempton (8). *G* is the glomerulus; *N*, the neck; *P*, the proximal tubule; *I*, the thin segment; *D*, the distal tubule; and *U*, the ureter.

Robinson flow counter, then allowed to decay for 15 days before assay for C^{14} (10). Total solute concentration was calculated (11) from the freezing point depression of a millimicroliter aliquot using Ramsay and Brown's ultramicro method (12).

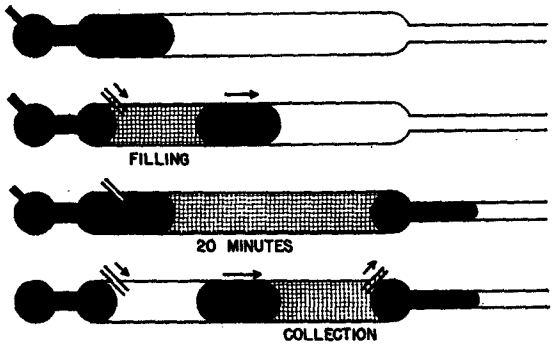

FIGURE 2. Schematic drawing showing technique of stopped flow microperfusion.

ELECTRICAL POTENTIAL DIFFERENCE MEASUREMENTS

In the experiments in which electrical potential difference measurements were performed, the method (Fig. 3) followed Giebisch's technique (13). After impalement of the tubule with the microelectrode, the electrical potential difference between the lumen of the tubule and the interstitial space was measured. The appearance of the tubular outline, following injection of the colored KCl was taken as evidence of intratubular location of the electrode. Electrical potential difference measurements were carried out both in tubules whose normal tubular fluid originated from the glomerulus, and

in perfused tubules whose tubular fluid composition was arbitrarily chosen as described before.

2. *Effect of 2,4-Dinitrophenol and Ouabain on Water Absorption*

The first group of experiments undertaken in our laboratory (Schatzmann, Windhager, and Solomon (14)) was a series of stopped flow microperfusions

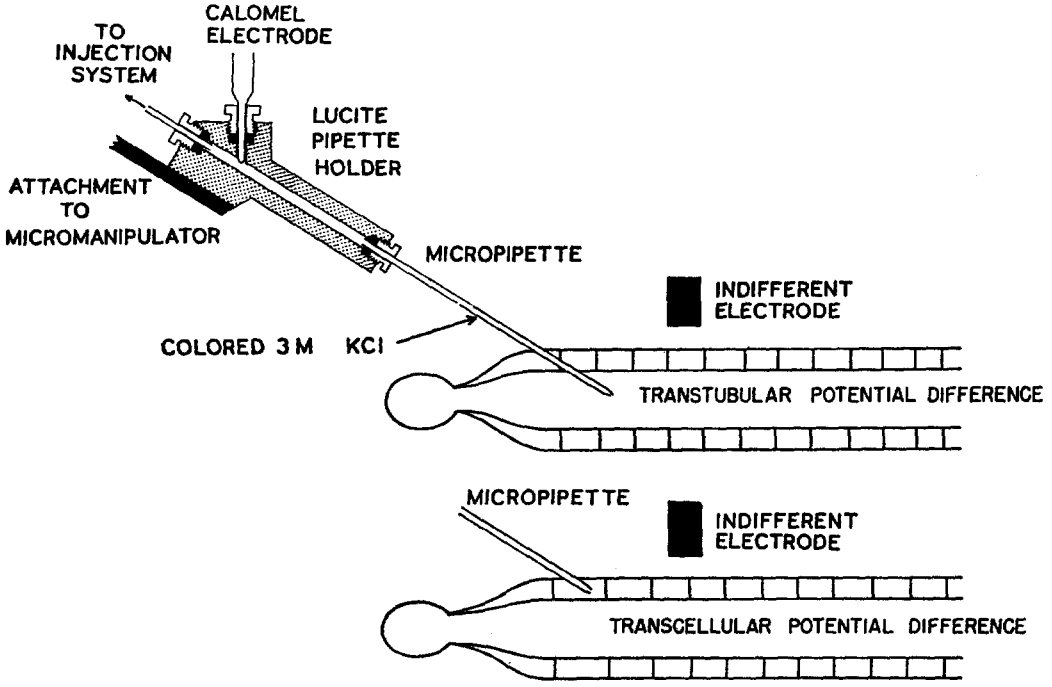

FIGURE 3. Schematic drawing showing technique of electrical potential difference measurement. Both electrodes are calomel electrodes connected to a high impedance voltmeter ($10^{14}\Omega$). The indifferent electrode is connected *via* a suitable agar bridge to the interstitial space of the animal. The explorer electrode is connected *via* a 3 M KCl solution (colored with chlorphenol red) to a micropipette (3 to 30 MΩ resistance). In the upper part of the drawing the location of electrodes for transtubular electrical potential difference is shown. In the lower one the location of the electrodes for measuring transcellular (outside wall of the cell, facing the interstitium) electrical potential difference is shown.

using a perfusion fluid isosmolar to the plasma of the *Necturus* (187 m.osM/liter), consisting exclusively of NaCl (100 m.eq./liter). Water movement was studied using C^{14} inulin. Schatzmann and colleagues (14) found that after 20 minutes, 27 per cent of the tubular fluid had been absorbed. This value is not statistically different from the 33 per cent absorbed by the proximal tubule when the tubular fluid is the normal glomerular filtrate.

In a further series of experiments, the same authors added 2,4-dinitrophenol to the perfusion fluid at a concentration of 2×10^{-4} M/liter. The water absorption was only 10 per cent. In a third series, ouabain at a concentration of 1.4×10^{-4} M/liter, also depressed water absorption to 10 per cent.

At that time, Giebisch (13) found a transtubular electrical potential difference of 20 mv., lumen negative to the interstitium, for tubules containing normal glomerular filtrate. Preliminary data from our laboratory gave the same value for the stopped flow microperfusions with 100 m.eq. NaCl/liter. Schatzmann and colleagues concluded from their experiments that water transport out of the proximal tubule was brought about by the active transport of Na. They based their conclusion on three assumptions: (1) isosmolar fluid absorption, (2) the absence of a significant transtubular pressure gradient, and (3) the absence of a transtubular Na concentration gradient.

3. Dependence of Water Movement on Intratubular Sodium Chloride Concentration

To further the study reported above, Windhager, Whittembury, Oken, Schatzmann, and Solomon (11) studied the relationship between intratubular NaCl concentration and net water movement. In this series of experiments, perfusion fluids of various NaCl concentrations were used, namely 100, 75, 62.5, and 50 m.eq. NaCl/liter. The chemical activity of the water was kept constant by adding mannitol, in the required amounts, to the perfusion fluid. FIG. 4 shows a plot of water movement (corrected for mannitol leakage) as a function of NaCl concentration. The shaded area shows that net transport of NaCl can take place against a considerable concentration gradient, since water can be transported from a solution containing 65 m.eq. NaCl/liter into one containing 100 m.eq. NaCl/liter. When the concentration gradient exceeds 35 m.eq. NaCl/liter the direction of the net movement is reversed and water enters the tubule. The apparently linear relationship between NaCl concentration in the lumen and the volume of water moved across the tubule wall demonstrates, therefore, that H_2O transport in the proximal tubule depends upon the tubular NaCl concentration rather than upon the water activity which was kept essentially constant. It was concluded (11) that water movement is secondary to NaCl movement and is, in whole or in part, a dependent process.

The question arose as to whether the dependence was total or partial; namely, whether the solute movement was sufficient to account for the total absorption of water, or whether other forces existed that would play a significant role in water movement. To answer this question, another series of experiments was performed by Windhager and colleagues (11). Isosmotic perfusion fluids containing 15, 50, and 100 m.eq. NaCl/liter were used (a

wider range of NaCl concentration than previously used). Measurements were made of the solute concentration of the injected and collected perfusion fluid and of the serum of the animals. The fluid absorbed was always found to be isosmotic with the serum. The average difference between the osmolarity

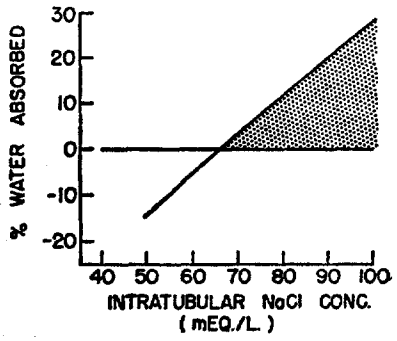

FIGURE 4. Water movement from proximal tubule as a function of initial intratubular NaCl concentration. Correction made for water movement resulting from mannitol diffusion. The shaded area corresponds to absorption of NaCl against a concentration gradient.

of the serum of the animal and that of the collected perfusate was only 2 mosM./liter with a standard error of 1.6 mosM./liter. These data justify the assumption of an isosmolar fluid absorption made by Schatzmann and colleagues (14) and referred to before.

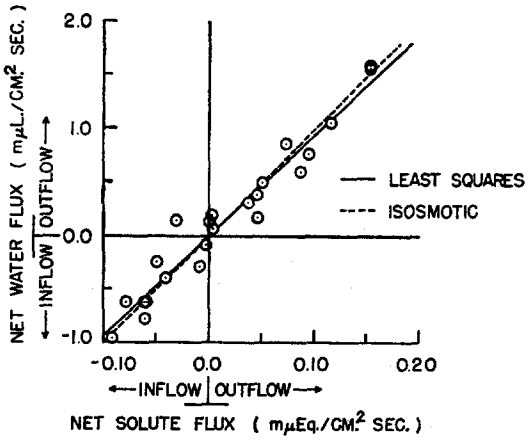

FIGURE 5. Net water flux as a function of net solute flux. The solid line has been drawn by the method of least squares, and the dashed line corresponds to the water movement predicted on the basis of isosmotic transfer of solute.

Fig. 5 shows the results of these experiments. The ordinate represents the net water flux as a function of net solute flux. Net water flux was calculated from water movement and volume and surface area of the proximal tubule; net solute flux was estimated from the solute content of the injected and collected perfusion fluids. The circles are the experimental values, and the

solid line has been calculated by the method of least squares. The dashed line shows the theoretical relationship expected from isosmotic transfer of solutes, which in the case of the *Necturus* is equivalent to 100 m.eq. NaCl/liter.

Three major conclusions may be drawn from Fig. 5: (1) The close relationship between the two lines provides visual evidence that the movement of solutes across the tubular wall is essentially isosmotic, in accord with the direct measurements by the freezing point method alone. (2) Isosmolar transport obtains over a wide range of water movement, from 54 per cent absorption to 35 per cent water entrance into the tubule. (3) The intercept of the regression line is not significantly different from zero; hence under our ex-

TABLE I

ELECTRICAL POTENTIAL DIFFERENCE MEASUREMENTS*

Initial tubular fluid composition‡	Potential differences	
	Transtubular	Transcellular
mM/liter	*mv.*	*mv.*
Normal§	−20.3 (70)‖	−73.2 (57)
100 NaCl	−20.3 (10)	
50 NaCl + 93 mannitol	−20.3 (9)	
10 NaCl + 168 mannitol	−20.0 (7)	
10 NaCl + 90 choline chloride	−6.6 (8)	−35 (4)
0 NaCl + 100 choline chloride	0 (6)	−31.5 (8)

* The electrical potential difference is measured with reference to the interstitial space of the animal.

‡ The composition of the perfusion fluid at the moment of the electrical potential difference measurements might have changed, for water and ion movements are likely to occur during the course of the experiments.

§ The tubular lumen containing the physiological, normal, glomerular filtrate.

‖ The number of experiments is given in parentheses.

perimental conditions, there is no net water flux when there is no net solute flux. Therefore, the only significant driving force for water movement arises from the net transport of solutes, largely NaCl. If any other force were important to the process, the intercept would be shifted from zero. Thus the absence of a significant transtubular pressure gradient assumed by Schatzmann and colleagues was justified. The finding of isosmotic transport and an intercept of zero shows that the transport of solute creates forces that are sufficient to account for the water movement. We therefore have to consider water movement as a secondary process, mediated solely by solute transport.

Concomitantly, transtubular electrical potential differences were measured in stopped flow microperfusions with different NaCl concentrations. Isosmolarity was maintained with mannitol. Table I shows the results of such experiments. It may be seen that the electrical potential difference is essentially the same (around 20 mv., the lumen being negative) for the tubules in which

the normal glomerular filtrate is circulating and for the stopped flow microperfusion experiments.

A correlation of these data leads to the conclusion that Na is transported against an electrical and chemical gradient and that no forces other than those arising from solute transport (essentially that of NaCl) are necessary to account for the water movement. These three conditions define the transport of Na from the proximal tubule as an active process.

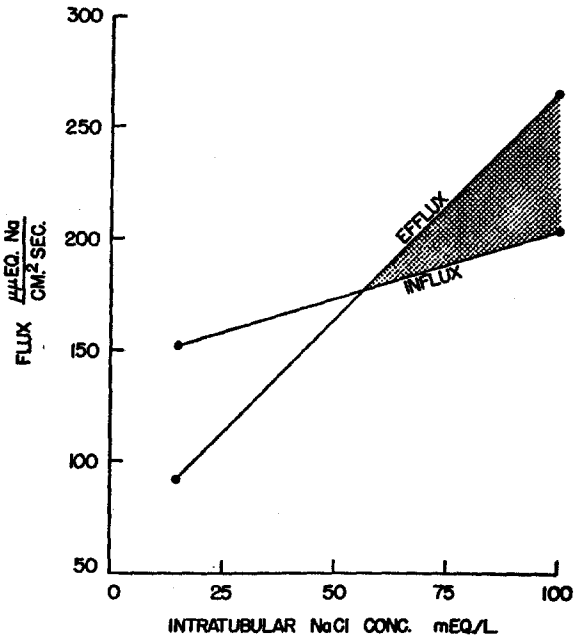

FIGURE 6. Efflux and influx of Na as a function of initial intratubular NaCl concentration. The shaded area is equivalent to the shaded area of Fig. 4; it corresponds to net Na movement against an electrochemical potential gradient.

THE SODIUM AND CHLORIDE FLUXES

A quantitation of the sodium fluxes as a function of Na concentration was then undertaken (10). Perfusion fluids containing 100 and 15 m.e.q NaCl/liter, respectively, were used; mannitol was added in requisite amounts to achieve isosmolarity. The results of this series of experiments gave confirmation to the third assumption of Schatzmann and colleagues (14) that no transtubular Na concentration gradient is introduced during perfusion with 100 m.eq. NaCl/liter.

Fig. 6 shows the fluxes as a function of the initial NaCl concentration. The shaded area corresponds to net sodium absorption against an electrochemical potential gradient. Fig. 7 shows the relationship between the observed fluxes

and the predicted flux ratio for independent passive diffusion according to Ussing's formula (equation 1) (5). It may be seen that for both Na concentrations, the observed ratio of the Na fluxes is higher than the predicted one. Therefore, provided the ions move singly and independently, the transport of Na is active by this criterion also.

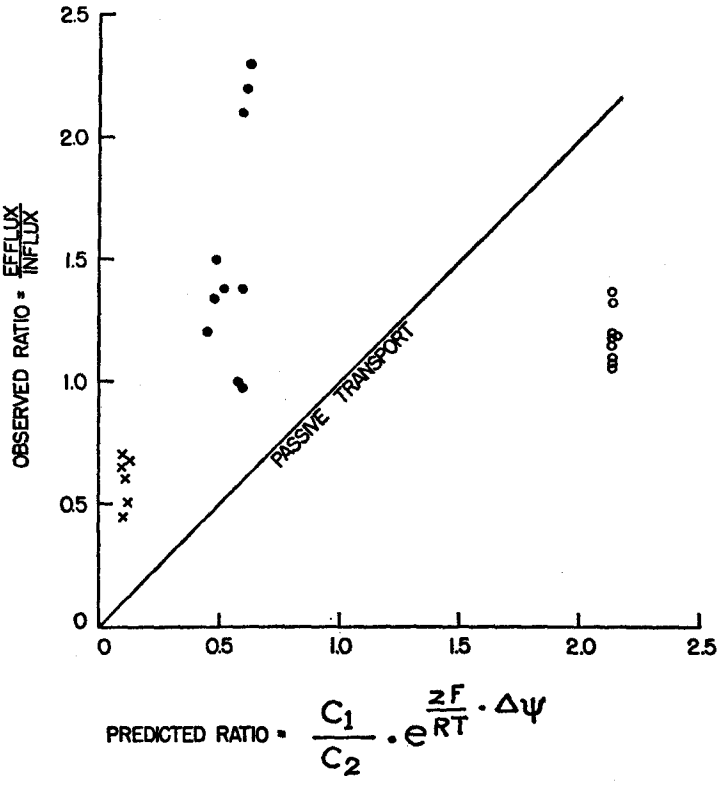

FIGURE 7. Ratio of efflux/influx found in the experiments, plotted as a function af the ratio predicted from Ussing's equation. The solid circles correspond to Na flux ratio in experiments in which the initial NaCl concentration was 100 m.eq./liter and the ×'s to Na flux ratios in experiments with 15 m.eq./liter in the perfusion fluid. The open circles are preliminary data on Cl flux ratios (15) studied, using isosmotic Ringer containing 98 m.eq.Na/liter and 74 m.eq. Cl/liter. It may be seen that the Na flux ratios lie above the line for passive independent transport while the Cl flux ratios lie below. C_1 and C_2 refer to the concentration of the ion in the lumen and interstitium respectively.

In contrast Fig. 7 also shows Giebisch and Windhager's data from preliminary experiments on Cl fluxes (15). For the chloride concentration they have studied, the observed ratio, 1.18, is lower than the predicted one, 2.14. Their points fall below the line for passive transport, as would be the case if exchange diffusion processes were involved.

4. Permeability Coefficient of the Proximal Tubule for Water

Another parameter which describes the characteristics of the tubular wall quantitatively is the permeability coefficient for water, the flow of water per unit force applied across a membrane of unit area. Thirty-two experiments were undertaken to determine this parameter (7). Osmotic pressure gradients were created by the addition of various amounts of mannitol to the perfusion fluid. The corresponding water fluxes were measured in the usual way. A value of 0.15×10^{-8} ml./(cm.^{2} sec. cm. H_2O of transtubular pressure gradient) was obtained for the permeability coefficient of the proximal tubule. This figure compares with the permeability coefficient of single cells, and is lower than the permeability coefficient of capillary membranes by some orders of magnitude (7).

It has been suggested (16, 17, 18) that the proteins circulating in the peritubular capillaries exert a pressure that could account for the reabsorption of water and subsequently of solutes from the proximal tubule. Our measured permeability coefficient for the proximal tubule permits the examination of the problem in a direct and quantitative way. The calculation shows that the proteins in the *Necturus* can only account for the absorption of a very small fraction (1.6 per cent) of the net water absorption observed (7).

This conclusion has been confirmed by direct experiments in which an isosmotic NaCl solution containing albumin was used as perfusion fluid. The colloid osmotic pressure of the perfusion fluid was 15 cm. of H_2O, 67 per cent higher than the average colloid osmotic pressure of the *Necturus* plasma, namely 9 cm. of H_2O. In these experiments the direction of the physiological protein osmotic pressure gradient was therefore reversed, yet the absorption of water continued in the usual direction (15 per cent of the tubular volume was absorbed from the lumen). This group of experiments proves that water absorption in the proximal tubule of the *Necturus* must be ascribed to causes other than the protein osmotic pressure (7).

5. Relationship between Tubular Electrical Potentials and Ion Flux

A large number of electrical potential difference measurements, both transtubular and transcellular, have been made under stopped flow microperfusion conditions. For these experiments, the initial composition of the perfusion fluid varied widely, though it was maintained isosmotic with plasma. The average results of some of these experiments are given in Table I and Fig. 8. Under normal conditions and in stopped flow microperfusions with 100 m.eq. NaCl/liter, the electrical potential difference across the luminal wall is 53 mv. while that across the outside wall is 73 mv. These values are similar

to those of Giebisch (13) for normal conditions and those obtained in the perfused *Necturus* kidney (22). The properties of the cellular membrane on the two sides are therefore different, and the cell is of the asymmetric type also found in frog skin (19). These observations lead to a number of attractive

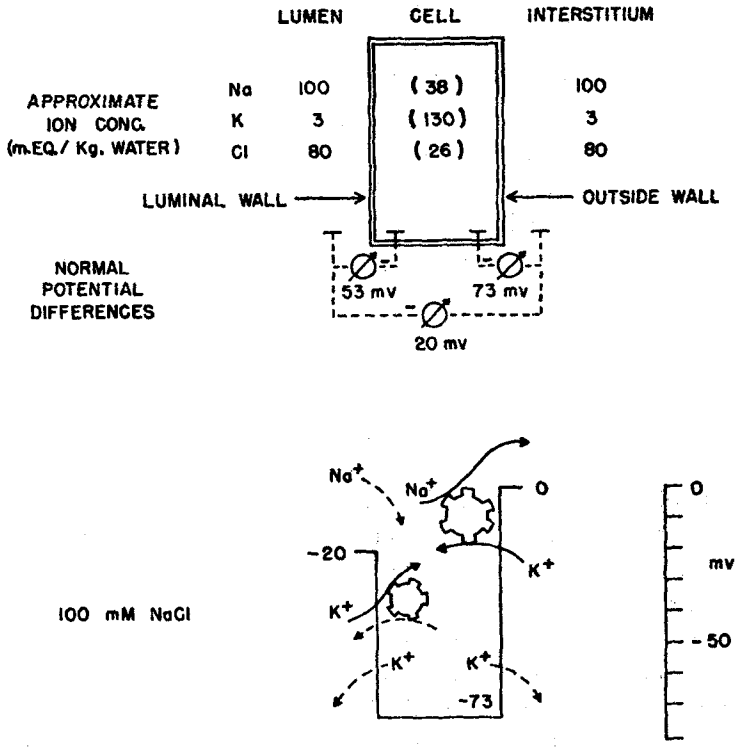

FIGURE 8. Schematic drawing showing the various electrical potential differences measurable in the proximal tubule. The Na, K, and Cl concentrations in cell water have been calculated from the data of Conway *et al.* (20) on frogs; they are given in parenthesis to show that they represent data from another species with a different total osmolar concentration. It can be seen that the normal transcellular electrical potential difference is 20 mv. (lumen negative); the transcellular (outside wall) electrical potential difference is 73 mv. (cell negative). So the electrical potential difference across the luminal wall is 53 mv. (cell negative).

speculations. It is possible to envision the position of the Na pump assuming that the Na concentration in cellular fluid is lower, whereas the K concentration is higher, than the plasma concentrations—as is the case for most cells. This assumption can be supported by a calculation of the intracellular concentrations on frog kidney, using the data given by Conway *et al.* (20) (see Fig. 8). From the lumen, the Na would enter the cell passively down its electrochemical potential gradient (see also reference 22); it would leave actively to the interstitium since both the electrical and the chemical po-

tential gradients at the outer wall are uphill. Thus it would seem sensible to place the Na pump at the outer cell wall in agreement with Pitts (21) and Giebisch (22). Since this wall has been shown by Giebisch (22) to be permeable to K, and essentially impermeable to Na, the pump would have to maintain the high intracellular K concentration against the leak of K from the cell to the interstitial space. Thus it seems reasonable to assume, as Ussing has done for the frog skin (19), that the pump exchanges Na for K. On this basis, the results obtained with choline chloride, as shown in Table I, are explicable. When Na in the lumen is replaced by choline, Na would leak from the cell into the lumen, and the reduced intracellular Na would cause the pump to slow down. The continuing loss of intracellular K would no longer be compensated by pump action, the intracellular K concentration would fall, and the electrical potential difference across the outer cell wall would thereupon decrease.

According to preliminary results of Giebisch the luminal wall is also permeable to K; in this case, however, the Na permeability is appreciable, and must play a role in the determination of the electrical potential difference across the wall (22). On this basis, the luminal wall electrical potential should also diminish in response to the fall in intracellular K in accord with the observations in Table I. Maizels and Remington (23) have shown that significant amounts of choline enter the tubular cells when kidney slices are exposed to choline-Ringer. It seems, therefore, reasonable to conclude that the luminal wall is also permeable to choline, as well as to Na. The interpretation of the changes in the luminal wall electrical potential is not as straightforward as that at the outer cell wall, being complicated by the presence of opposing choline and Na diffusion potentials. It should be pointed out that these speculations upon the electrical potential changes resulting from the substitution of choline for Na in the perfusion fluid, rest upon the assumption that the permeability of the two cell walls remains unchanged in the presence of choline.

The observed electrical potential differences also lead to conclusions about the nature of the K transport process. The results of Bott (24) indicate that the proximal tubular K concentration does not rise above the concentration observed in the plasma. As some 30 per cent of the glomerular filtrate water is absorbed during passage down the proximal tubule, Bott's observations demonstrate net absorption of K in the proximal tubule. Since the tubular concentration is no greater than that in the peritubular fluid, and since there is an opposing electrical potential gradient, K transport would be an active process. In this case, it would seem more likely that the pump would be placed at the inner wall, since the measured electrical potential difference at that wall is not sufficient to balance the concentration gradient, whereas the results of Giebisch (22) indicate a near-equilibrium distribution of K at

the outer wall. This inner wall pump could either pump K into the cell, or extrude Na into the lumen in an exchange for K. In the first possibility the electrochemical potential gradient to overcome would be smaller. The inner wall K pump need only be sufficiently powerful to overcome a 35 to 45 mv. electrochemical potential gradient. This may be contrasted with the 90 to 110 mv. gradient which the Na pump must overcome if it is located at the outer wall.

In summary, evidence has been presented from studies in the proximal tubule of the kidney of *Necturus*, that enables us: (1) to define the transport of Na as an active process in contrast to Cl which is passive; (2) to explain the movement of water as entirely due to forces arising from the transport of solutes; (3) to demonstrate that the colloid osmotic pressure of the plasma proteins does not account for any important fraction of the water movement in this animal; and (4) to make suggestions concerning the intracellular location of the Na and K pumps.

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