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Anion Permeation in the Proximal Tubule of *Necturus* Kidney: The Shunt Pathway

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Summary. The effect of foreign anions on transepithelial potential difference and transepithelial input conductance was studied in the isolated perfused *Necturus* kidney. Two microelectrodes (recording and current-injecting) were inserted into the lumen of single proximal tubules and the peritubular perfusate was shifted reversibly for 30–60 sec from a physiologic Ringer's solution to a test solution in which chloride was replaced isosmotically by a foreign anion. The permeability sequence, obtained by potential measurements, was: lactate < glutamate < gluconate < pyruvate < benzene sulfonate ≦ acetate ≦ F < propionate < BrO_3 < formate < ClO_3 < Cl < ClO_4 < I ≦ Br < NO_3 < SCN. Transepithelial conductance decreased when the tissue was perfused with anions less permeable than chloride but the conductance sequence was different from the permeability sequence. Such discrepancies were more pronounced during perfusion with hyperpolarizing anions; ClO_4^- and I^- (both more permeable than chloride) produced an important decrease in transepithelial conductance, followed by incomplete reversibility when the perfusion was shifted again to chloride Ringer's. The results are best explained by the presence of weak positive fixed charges, governing anion permeation, at the shunt pathway of the proximal tubule. An analysis of the data allows tentative estimates of shape and size of the sites.

In a previous study designed to establish a quantitative relationship between bi-ionic potentials and permeability properties of single membranes in the proximal tubule of *Necturus* kidney, it was found that the replacement of extracellular chloride by benzene-sulfonate produced a shift of intraluminal potential in the positive direction (Anagnostopoulos, 1973*a*). No attempt was made at that time to get further insight into the mechanisms governing anion permeation across the shunt pathway.

In recent years, various mechanisms of ion permeation have been postulated and several investigators have stressed the peculiarities of anion permeation in various tissues (Hutter & Noble, 1961; Del Castillo, De Mello & Morales, 1964; Hagiwara & Takahashi, 1967; Hagiwara, Toyama & Hayashi, 1971; Takeuchi & Takeuchi, 1971; Hagiwara &

Takahashi, 1974). With this background in mind we have investigated the behavior of the shunt pathway in the perfused *Necturus* kidney during anionic substitutions. Chloride was replaced by a number of inorganic and organic anions in extracellular fluid and the effects of such substitutions on transepithelial p.d. and transepithelial input conductance were studied. It was found that permeability and conductance sequences were not strictly identical, especially when anions more permeable than chloride were studied. Moreover, the decrease in conductance induced by some of the hyperpolarizing anions did not appear fully reversible when the test anion was removed from the perfusion fluid.

Materials and Methods

Preparation

The technique for preparation of the animals was essentially similar to that described by Giebisch, Sullivan and Whittembury (1973). Briefly, after anesthesia by immersion in 1.5% tricain solution, the peritoneal cavity was opened via a parasagittal incision running from the cloaca to mid-thoracic level. The iliac, intestinal and lower genital vessels were tied, but no protease was applied on the surface of the kidneys as in a previous study. The animals were decapitated and pithed. The tail was cut. Subsequently, the portal system was catheterized via the caudal vein and another catheter was inserted into the aorta. The caval outflow was diverted away from the preparation through a third large catheter placed at the rostral end of the kidneys.

Perfusion

The composition (mM/liter) of the control Ringer's solution was: 95.0 NaCl, 4.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 6.8 tris, 5.0 NaOH, 2.2 glucose. In addition, PVP (20 g/l) was added to provide for colloid. In the substituted solutions, NaCl was replaced isosmotically by the sodium salts of the following anions: F^-, Br^-, I^-, NO_3^-, BrO_3^-, ClO_3^-, ClO_4^-, SCN^-, formate, acetate, lactate, propionate, pyruvate, glutamate, gluconate and benzene sulfonate. Such substitutions brought about a decrease of Cl_0 by a factor of 10. Since the magnitude of bi-ionic potentials may be affected by pH (Anagnostopoulos, 1972), fresh solutions were made every day and their pH was measured and, when necessary, adjusted at pH 7.4, by slight modifications of the tris/NaOH ratio.

Both, aorta and portal vein, were perfused with the control Ringer's solution. A multi-way stop-cock system was placed at 3 cm from the end of the portal vein catheter, allowing thus for changes in the composition of peritubular perfusate. It was possible to replace Cl^- by six different anions in each experiment. Constant perfusion pressure was achieved by means of reservoirs placed at 10–15 cm (portal vein) and 20–25 cm (aorta) above the kidneys. The respective flow rates were 1–3 ml/min and 1.5–5 ml/min. Such high flow rates are known to induce volume expansion in the perfused *Necturus* kidney (Bentzel, Anagnostopoulos & Pandit, 1970) but they appeared necessary to achieve rapid changes in the composition of peritubular perfusate during ionic substitutions.

The exposure of the tissue to a foreign anion never was longer than 1 min. After 2–5 hr of perfusion, the responses (i.e. bi-ionic potentials) became smaller in amplitude and slower in time course. This was probably due to a decrease in perfusion flow rates of superficial

peritubular capillaries, as evidenced by occasional injections of 0.05 ml of 0.01% lissamine green (LG) solution in portal circulation. In our experimental set-up transit times of appearance of LG into peritubular capillaries longer than 6 sec were considered as inadequate and the experiment was discontinued. The surface of the kidneys was always kept moist by slowly dripping a PVP-free physiologic Ringer's solution (pH 7.4). All impalements were performed at early or middle convolutions, namely in the portion of the proximal tubule lying between the glomerulus and the external border of the kidney, before the point where the nephron bends heading towards the median line.

Recording

Transepithelial p.d. recordings were made between a 3 M KCl microelectrode (resistance 15–40 MΩ) introduced into the lumen of a proximal tubule and an indifferent 3 M KCl agar electrode placed in the peritoneal cavity. They were connected through AgCl:Ag half-cells to a Medistor (type A-35) cathode follower; its output was recorded by a Watanabe linear pen recorder (type WTR 281). Bi-ionic potentials could be measured with this arrangement during changes of extracellular fluid. To estimate, in addition, changes of transepithelial input conductance, a second microelectrode was inserted into the lumen of the same tubule. An accurate determination of interelectrode distance could not be achieved since the microelectrodes were flexible, their tips were out of sight when they advanced inside the superficial fluid, and the epithelial layer appeared deformable during impalement. However, from their visible portion, the distance of the tips was roughly estimated, in most cases, at 100–150 μm.

The second microelectrode could be connected either to a cathode follower (Medistor, A-35) or to a voltage stimulator (Electromed, France). The indifferent electrode of this circuit was grounded. The experimental set-up was similar to that described previously (Anagnostopoulos & Velu, 1974, Fig. 1) except for two modifications: (a) depolarizing voltage pulses were delivered through a 1000 MΩ resistor (instead of 120 MΩ) and (b) in the present set of measurements current intensity was always 10^{-7} A (microelectrode resistance neglected). Constant current pulses were delivered every 4 sec and lasted 1 sec. Since the proximal tubule is a leaky epithelium (Windhager, Boulpaep & Giebisch, 1967; Boulpaep, 1972; Anagnostopoulos & Velu, 1974), and the resistance of the thin layer of superficial fluid may not be negligible as compared to transepithelial resistance, the measurements were carried out as follows: the stimulating electrode was inserted into the lumen and constant current pulses were delivered through it. The amplitude of electrotonic potential was determined when the recording tip was (a) into the lumen and (b) at the surface of the kidney; the data were corrected accordingly and the figures given below are the corrected ones. The control conductance in Ringer's solution g_{Cl} was measured before applying each test solution and after returning to Cl solution from the test solution; when the difference between these two values was less than 5% and the change did not occur in the same direction in all experiments, their mean was taken as control transepithelial conductance. Two anions (I^- and ClO_4^-) produced a significant decrease in transepithelial conductance and the reversibility was incomplete after returning to perfusion with Ringer's solution; in these cases the control conductance g_{Cl} was taken as the initial value, before perfusion with the test solution.

Junction Potentials

Since the reference electrode was in the peritoneal cavity and the caval outflow was diverted in another direction (away from the preparation), junction potentials could arise when chloride was replaced by foreign anions in the peritubular circulation. Their amplitude was estimated in most cases as follows: just before or after recording of transepithelial bi-ionic

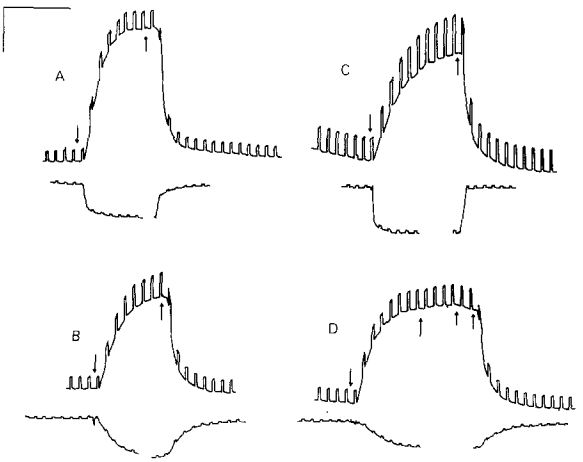

Fig. 1. Effects of peritubular perfusion with some of the "depolarizing" anions on transepithelial p.d. and transepithelial input conductance. *A*: lactate; *B*: benzene sulfonate; *C*: glutamate; *D*: acetate. In each tracing the first arrow indicates the beginning of perfusion with the foreign anion and the last one recovery to Ringer's solution. In tracing *D*, potassium concentration was increased by a factor of 10 (part of Na-acetate being replaced by K-acetate; second arrow); the perfusion was shifted again to Na-acetate (third arrow) and finally to Ringer's solution. Each pair of tracings comprises one recording obtained with two intraluminal microelectrodes *(top)* and a second one *(bottom)* in which the current-injecting electrode was left into the lumen but the recording tip was moved into the interstitium. The lower tracings were used to correct ΔV for junction potentials and also to correct the amplitude of electrotonic potentials, by taking into account surface fluid resistance. Comparison of luminal and superficial recordings shows clearly that there is no possibility of mistaking the localization of the recording tip. In tracings *C* and *D* the baseline before perfusion with foreign anions is not horizontal due to incomplete recovery in p.d. from previous substitutions. Scale: 10 mV × 30 sec. Shifts in p.d. in the positive direction, upwards

potentials, the microelectrode was inserted into the adjacent interstitium and peritubular substitutions of Cl^- were performed in the same sequence and for about the same duration of time. The amplitude of junction potentials, as obtained from recordings with interstitial insertion of the tips, was substracted from transepithelial bi-ionic potentials. Some examples of paired recordings (bi-ionic and junction potentials) appear in Figs. 1 and 2. In a few cases, these verifications were not performed; corrections were made by using the mean value of junction potentials obtained in all other experiments. The average amplitude of junction potentials, when tissue exposure to foreign anions was no longer than 1 min, was (mV): −10.2 gluconate, −9.2 glutamate, −7.8 benzene sulfonate, −7.1 pyruvate, −7.0 lactate, −6.5 propionate, −5.8 acetate, −4.2 F^-, −3.0 BrO_3^-, −2.7 formate, −2.2 ClO_3^-, −2.2 ClO_4^-, −2.0 SCN, −1.8 I^-, −1.1 NO_3^- and +0.5 Br^-. The SEM was never greater than 0.2 mV. Some of the above figures may include an error of up to ±1.0 mV due to tip potentials, as estimated *in vitro* with a separate set of microelectrodes. This error is within the limit of experimental variations observed from one animal to another and from tubule to tubule in the same *Necturus*. Unless otherwise stated the data are given, after correction for junction potentials, as mean ±SD.

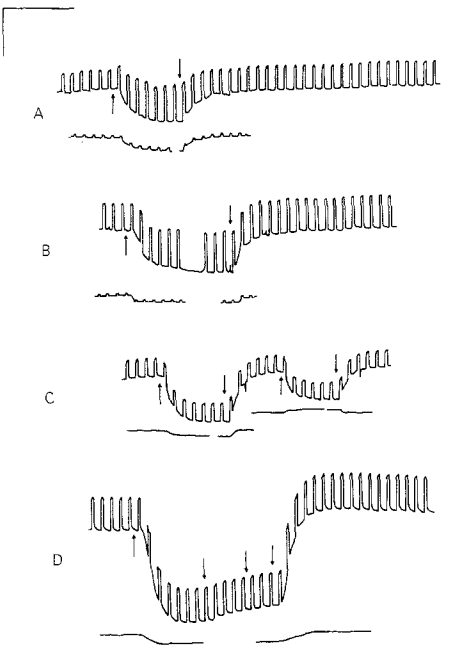

Fig. 2. Effects of peritubular perfusion with some of the "hyperpolarizing" anions on transepithelial p.d. and transepithelial input conductance. Symbols and conventions as in Fig. 1. *A*: ClO_4^-; *B*: I^-; *C*: NO_3^- (first arrow), Ringer's solution (second arrow), Br^- (third arrow), Ringer's solution (fourth arrow); *D*: SCN^-; the second and third arrows indicate the passage from NaSCN to KSCN and back to NaSCN by a reversible increase of K_o from 4.5 to 45 mM. Note a transitory p.d. shift in the positive direction during recovery to Cl^- Ringer's from NaSCN

Results

Do Transepithelial Bi-ionic Potentials Reflect the Properties of the Shunt Pathway Alone?

In previous studies it has been emphasized that bi-ionic potentials in epithelial tissues must be analyzed by means of an equivalent electrical circuit (Anagnostopoulos, 1973*a*, *b*); the recorded shifts in p.d. resulting from extracellular substitutions are not related to the properties of a single membrane but they may be influenced by the conductances and emf's of the three diffusion barriers of the epithelium. However, the proximal tubule of *Necturus* kidney represents a privileged biological structure, in which the sum of cell membrane resistances in series is about two orders of magnitude greater than the shunt resistance (Windhager *et al.*, 1967; Boulpaep, 1972; Anagnostopoulos & Velu, 1974). Under these circumstances, it is intuitively expected and it can be rigorously

demonstrated by circuit analysis (Anagnostopoulos, 1973*a*), that the recorded changes in transepithelial p.d. during extracellular bi-ionic substitutions do reflect the actual changes in p.d. due to the properties of the shunt pathway alone, without significant distortion from other parts of the circuit. The same does not apply to peritubular membrane recordings.

Bi-anionic Potentials

The amplitude of transepithelial potential change ΔV during extracellular bi-anionic substitutions, was measured for each test anion at its maximum. All organic anions tested but SCN^-, as well as F^-, BrO_3^- and ClO_3^- produced a significant shift of luminal p.d. in the positive direction, suggesting that the permeability ratio P_A/P_{Cl} was smaller than unity for these anions. A different situation prevails for the anions Br^-, I^-, NO_3^-, ClO_4^- and SCN^-. The magnitude of bi-anionic potentials ΔV, after correction for junction potentials, appears in Table 1, column 2. Some representative recordings are shown in Figs. 1 and 2.

The change in p.d., ΔV, is related to transepithelial ionic permeabilities by the Goldman-Hodgkin-Katz equation, in the following form:

$$\Delta V = -\frac{RT}{F} \ln \frac{P_{Cl}(Cl_0)_{exp} + P_A(A_0)_{exp} + P_{Na}(Na_i)_{exp}}{P_{Cl}(Cl_0)_{cont} + P_{Na}(Na_i)_{cont}} \quad (1)$$

where the subscripts $_{cont}$ and $_{exp}$ refer to ionic concentrations during perfusion with physiologic Ringer's and test solution, respectively. Potassium contribution is neglected since its luminal and extracellular concentrations are 20 times smaller than those of Na^+ (Giebisch, 1956; Bott, 1962). In addition, the shunt pathway is not selective to K^+ and Na^+ (Anagnostopoulos, 1973*a*); thus, Na_i could be adequately corrected to represent the luminal concentration of monovalent cations.

Eq. (1) does not allow a precise estimate of the P_A/P_{Cl} ratio, since the cationic permeability of the shunt pathway may not be negligible; nor does it allow an estimate of P_{Na}/P_{Cl}, since little is known on the permeability of foreign anions. However, it is possible to estimate an upper limit for the ratios P_A/P_{Cl} and P_{Na}/P_{Cl}, by assuming total impermeability for Na^+ or for substituting anions, respectively. Obviously, such assumptions appear unrealistic, but the corresponding estimates are extremely useful since they set the absolute boundaries within which lay the values of P_{Na}/P_{Cl} and of the various P_A/P_{Cl}'s: (a) The *upper limit* of P_A/P_{Cl} for each depolarizing anion is listed in the upper part (the first 11 anions) of Table 1, column 3;

Table 1. Summary of complete data on permeability and conductance measurements

	1 N	2 ΔV (mV ± SD)	3 P_A/P_{Cl}	4 P_{Na}/P_{Cl}	5 $\frac{V_A}{V_{Cl}}$	6 g_A/g_{Cl}	7 u_A/u_{Cl}	8 K^A_{Cl}
Lactate	7	28.9 ± 4.1	0.24	0.33	1.64 ± 0.10	0.37	0.18	1.28
Glutamate	6	28.3 ± 3.1	0.25	(0.34)	1.78 ± 0.22	0.32	0.14	1.79
Gluconate	6	27.3 ± 1.5	0.26	(0.37)	1.85 ± 0.17	0.29	0.12	2.17
Pyruvate	7	26.9 ± 4.8	0.27		1.69 ± 0.24	0.35	0.16	1.69
Benzene sulfonate	10	26.1 ± 5.3	0.28		2.01 ± 0.15	0.25	0.096	2.92
Acetate	12	26.0 ± 3.6	0.28		1.59 ± 0.14	0.40	0.20	1.40
F^-	6	25.9 ± 1.4	0.28		1.66 ± 0.21	0.36	0.17	1.65
Propionate	4	24.5 ± 1.3	0.30		1.73 ± 0.18	0.33	0.15	2.00
BrO_3^-	4	15.0 ± 0.9	0.50		1.48 ± 0.05	0.46	0.25	2.00
Formate	3	10.2 ± 1.3	0.62		1.18 ± 0.07	0.72	0.54	1.15
ClO_3^-	8	3.6 ± 1.3	0.85		1.25 ± 0.06	0.64	0.43	1.98
ClO_4^-	13	−4.0 ± 1.2	1.19		1.95 ± 0.20 (1.24 ± 0.09)	0.26 (0.65)	0.10 (0.44)	11.9 (2.70)
I^-	10	−5.8 ± 1.3	1.28		1.40 ± 0.11 (1.11 ± 0.08)	0.51 (0.81)	0.29 (0.66)	4.41 (1.94)
Br^-	10	−6.2 ± 1.1	1.31		0.92 ± 0.05	1.18	1.41	0.93
NO_3^-	7	−8.7 ± 1.7	1.45		1.05 ± 0.05	0.91	0.82	1.77
SCN^-	8	−14.6 ± 2.7	1.87		1.00 ± 0.06	1.00	1.00	1.87

N: number of observations; ΔV: change in transepithelial p.d. during replacement of extracellular Cl^- by foreign anions; hyperpolarizations are given a negative sign; V_A/V_{Cl}: relative change in the amplitude of electrotonic potential (transepithelial), during anionic substitutions. Columns 2 and 5 refer to experimental determinations. The ratio g_A/g_{Cl} (column 6) derives directly from V_A/V_{Cl} according to Eq. (2). The "limiting" values of P_A/P_{Cl}, P_{Na}/P_{Cl}, u_A/u_{Cl} and K^A_{Cl} (columns 3, 4, 7 and 8, respectively) were calculated by making appropriate assumptions *(see text)*; they represent, at best, close approximations. The figures in parentheses in the ClO_4^- and I^- lines correspond to residual changes in electrotonic potential (and subsequent determinations directly related to it) during recovery to Ringer's perfusion after exposure to the test solution.

it was calculated according to Eq. (1) by making the term P_{Na} nul. The actual value of each P_A/P_{Cl} is certainly smaller than that indicated in Table 1, but the permeability sequence is correct. (b) The least permeable among depolarizing anions were lactate and glutamate. From the magnitude of depolarization induced by lactate, the *upper limit* of P_{Na}/P_{Cl} may be estimated at 0.33. Eq. (1) was used for that purpose, by assuming $P_A = 0$. (c) The permeability ratio P_A/P_{Cl} for hyperpolarizing anions was calculated as that for depolarizing ones. It is given in the lower part of column 3, in Table 1. It indicates the sequence of anionic permeabilities and the *lower limit* which can be ascribed to each P_A/P_{Cl} ratio.

Transepithelial Conductance Determinations During Anionic Substitutions

The proximal tubule of *Necturus* kidney has been reported to display cable properties (Windhager *et al.*, 1967; Boulpaep, 1972). If the input transepithelial conductance is designed by g_{Cl} in the control state and by g_A during perfusion with foreign solutions, the ratio g_A/g_{Cl} can be estimated by comparing the steady-state electrotonic potential obtained in tubules bathed with Cl-Ringer's and test anion solutions, V_{Cl} and V_A respectively:

$$g_A/g_{Cl}=(V_{Cl}/V_A)^2. \tag{2}$$

Eq. (2) may be applied to the present work, assuming that (a) the geometry of the tubule and the position of the tips did not vary during extracellular substitutions, (b) the internal resistance of tubular fluid did not change appreciably within one minute of tissue exposure to foreign anions, and (c) interelectrode distance, though small as compared to the space constant of 260 μm (Boulpaep, 1972) was sufficient to fit the linear portion of the curve relating the logarithm of electrotonic potential to distance. Eq. (2) derives directly from cable equations provided that the above assumptions are met (Ohashi, 1970).

Since the amplitude of responses may vary from tubule to tubule, conductance and permeability ratios were always compared in paired experiments. In the group of depolarizing ions (eight organic anions, F^-, BrO_3^- and ClO_3^-) where $P_A/P_{Cl}<1$, the conductance ratio g_A/g_{Cl} was also smaller than unity but the sequences were not identical (compare columns 3 and 6 in Table 1). Such discrepancies were more pronounced with the hyperpolarizing anions, except for Br^-; I^-, NO_3^-, ClO_4^- and SCN^- did not increase the transepithelial conductance as one would expect. There was no correlation between the magnitude of hyperpolarization and the direction or the magnitude of change in conductance.

The complete data on g_A/g_{Cl} are given in Table 1, column 6, and some representative recordings are shown in Figs. 1 and 2.

Reversibility of p.d. and Conductance Changes

In general, the above described changes in p.d. and input conductance were reversible. However, upon removal of the foreign anion from extracellular fluid and perfusion with the control Ringer's solution, the recovery in p.d. was, on occasion, followed by an overshoot. The amplitude of the overshoot was small, compared to that of the bi-ionic potential preceding

it, but its disappearance was slow, requiring up to 2 or 3 min. The disappearance of the overshoot was slower with depolarizing anions (lactate, glutamate, gluconate, etc.) than with the hyperpolarizing ones (SCN^-). These observations are consistent with some exchange between Cl^- and foreign anions in luminal fluid and transient reversal of the anionic gradient during recovery.

More interestingly, some changes in input conductance did not appear to be fully reversible within a reasonable amount of time. This occurred only with two hyperpolarizing anions, ClO_4^- and I^-; both produced small hyperpolarization (as compared to that of SCN^- and NO_3^-). Perfusion of peritubular fluid with ClO_4^- decreased transepithelial input conductance by a factor of four (average of 13 determinations; exposure of the tissue to ClO_4^- less than 45 sec), when the p.d. shifted in the negative direction by only 4.0 mV. Upon recovery to control Ringer's perfusion, transepithelial p.d. returned to its initial value within a few seconds, but transepithelial input conductance was still smaller than control by a factor of nearly two and stabilized at this level for more than 2 min. An example of such changes is shown in Fig. 2. The g_A/g_{Cl} ratio before, during and after perfusion with I^- was 1, 0.5 and 0.8.

Other Findings

Six tubules were exposed for 30 sec to the sodium salt of acetate, SCN^- or ClO_4^-, then for another 20–30 sec to the potassium salt of the same ion, followed by perfusion with Ringer's. The shift of perfusing solution from Na^+ to K^+ did not affect transepithelial p.d. by more than one mV, nor did it produce appreciable changes of transepithelial conductance (Figs. 1 and 2). It is concluded that the lack of cationic selectivity of the shunt pathway, already established in the control state (Anagnostopoulos, 1973*a*), persists during most anionic substitutions. It is also likely that the observed changes in transepithelial conductance during anionic substitutions are not related to variations of cationic conductance.

Three tubules, perfused with benzene sulfonate, ClO_4^- and acetate were tested for the effect of hyperpolarizing pulses. The changes in the amplitude of the electrotonic potential (not included in the statistics) were within the range of variations observed with depolarizing current.

Transepithelial p.d.

Although this study was not aimed at measuring the precise magnitude of transepithelial p.d., its average value could be estimated from available

recordings at -0.8 ± 0.8 mV. The change in the amplitude of electrotonic potential when the voltage-measuring electrode was advanced from interstitium into the lumen, as well as bi-ionic potentials subsequently achieved by peritubular perfusion with foreign anions, indicate that the localization of the tip in the lumen was correct and the impalements did not produce significant leaks (Figs. 1 and 2). However, the estimate of transepithelial p.d. comprises an error of up to 1 mV, due to tip potentials which may arise when KCl electrodes rather than Ringer's-filled tips are used for transepithelial determinations (Frömter & Gessner, 1974). Thus, our mean of -0.8 mV should be taken only as a close approximation of the true transepithelial p.d. It should be recalled that the value of -0.8 mV is at variance with other reports on transepithelial p.d. To test whether this discrepancy is the result of some alteration of the tissue, due to the perfusion with artificial solutions, a thorough investigation was undertaken independently in the intact *Necturus* kidney; such experimental determinations, as well as various theoretical considerations fully support the present data (Edelman & Anagnostopoulos, 1975; also *in preparation*).

Discussion

A lack of correlation between the permeability sequence of tested anions and concomitant changes in membrane conductance, when Cl_0 is replaced by foreign anions, has been reported in various tissues (*see*, for references: Takeuchi & Takeuchi, 1971; Hagiwara *et al.*, 1971; Hagiwara & Takahashi, 1974). Three mechanisms are generally considered to account for such discrepancies: (a) The foreign anion may alter cationic conductances; P_A/P_{Cl} ratios greater than unity are consistent with an overall decrease in conductance if cationic conductance(s) undergo a sharp decrease. (b) The foreign anion may reduce Cl^- permeability. Reduction in Cl^- efflux when Cl_0 is replaced by other anions has been observed in several tissues (Harris, 1958; Adrian, 1961; Spurway, 1965; Hutter & Warner, 1967; Moore, 1969). However, to account for a decreased membrane conductance, one has to assume that the presence of the anion A^- in the membrane decreases the combined anionic conductance, even if a concomitant hyperpolarization indicates that $P_A > P_{Cl}$ (or $P_A = P_{Cl}$ if there is no p.d. change). (c) Anion permeation occurs through fixed positively charged pores (Conti & Eisenman, 1965). In such structures, the permeability ratio P_A/P_{Cl} may be greater than unity, when $g_A/g_{Cl} < 1$ and $u_A/u_{Cl} < 1$, provided that the value of the affinity constant K_{Cl}^A is sufficiently high (*see* Eq. (3) in the Appendix; u_A, u_{Cl} and K_{Cl}^A are also defined in the Appendix).

The data of this paper will be discussed at first with regard to the fixed-charge theory. Eisenman (1965) has predicted the halide anion permeability sequence across positively charged membranes, as a function of site field strength. Our data ($P_{Br} \simeq P_I > P_{Cl} > P_F$) are consistent with weak positive sites at the shunt pathway; the low magnitude of selectivity ratios (compare the halide permeability ratios in *Necturus* to the ratio of the corresponding limiting equivalent conductivities in free solution, Table 2) suggests that the number of water molecules in the vicinity of the interacting positive sites and mobile anions is rather high (Eisenman, 1965; Diamond & Wright, 1969).

As stated earlier there is no satisfactory method to determine with accuracy the permeability ratio P_A/P_{Cl} in the proximal tubule of *Necturus*. Similar limitations apply to the mobility ratio u_A/u_{Cl}. For the sake of comparison, however, u_A/u_{Cl} and K_{Cl}^A were computed from the figures of P_A/P_{Cl} and g_A/g_{Cl} according to Eqs. (3) and (4). They appear in Table 1, columns 7 and 8, respectively. Since the various P_A/P_{Cl}'s are only tentative figures (upper or lower limits), the corresponding u_A/u_{Cl}'s and K_{Cl}^A's cannot represent anything beyond this. They should be taken at best as an indication of sequence.

The estimated figures for K_{Cl}^A are relevant to the observations made during perfusion with the hyperpolarizing anions I^- and ClO_4^-. The corresponding binding constants are large enough to account for high permeabilities with respect to chloride, despite low ionic mobilities within the membrane (Table 1). Transepithelial conductance decreased during perfusion with I^- and ClO_4^-; it remained consistently below control levels when the perfusion was shifted from the test solution to Ringer's solution, despite total reversibility of p.d. changes. This observation can be readily explained if the affinity constant of positive sites for I^- and ClO_4^- is quite high. Binding to and neutralization of positive sites by anions reduces the fixed-charge density of the conductive channels and decreases accordingly transepithelial conductance (*see* Conti & Eisenman, 1965; Hagiwara *et al.*, 1971). Strong bonds between sites and counter-ions may not be broken off completely when the foreign anion is removed from the perfusing solution; the resulting delayed neutralization of some sites is consistent with incomplete conductance reversibility after returning from the test solution to Ringer's perfusion. To our knowledge similar observations have never been reported in biological structures; they may be the best experimental evidence supporting Eisenman's view of fixed positive charges governing anion permeation, through the product of membrane mobility ratio u_A/u_{Cl} times the binding constant K_{Cl}^A.

Table 2. Some physical properties of anions

	1 Naked size of ions or polar groups (Å)	2 Hydrated size (relative to K^+ ions)	3 Hydration energy (Kcal/g ion)	4 Limiting equivalent conductivities in water at 25 °C (cm^2/Ω equiv)
ClO_4^-	(2.92) $\{r_1=2.90\}$ $\{r_2=2.83\}$	1.09	54	67.3
I^-	2.16	0.96	49	76.8
Br^-	1.95	0.94	63	78.1
Cl^-	1.81	0.96	67	76.35
F^-	1.36	1.33	94	55.4
BrO_3^-	(2.38) $\{r_1=3.18\}$ $\{r_2=3.12\}$	1.32	68	55.7
ClO_3^-	(2.16) $\{r_1=2.97\}$ $\{r_2=2.86\}$	1.14	61	64.6
NO_3^-	(1.98) $\{r_2=2.64\}$	1.03	55	71.5
SCN^-		1.11	45	
SO_3^-	$\{r_1=2.80\}$ $\{r_2=2.73\}$			
COO^-	$\{r_3=2.67\}$			
Formate		1.35		54.5
Acetate		1.80		40.9
Propionate		2.05		35.8

Naked ion sizes for halide ions were calculated from their crystal radii. Those for oxyanions, given in parentheses, were computed by Araki *et al.* (1961); they represent the radius of a sphere, equivalent to the volume of each oxyanion, as estimated by $0.25\,nr$, where n is the number of arms extending from the central (nonpolar) atom and r their length, i.e. the interatomic distance between Cl, Br or N, respectively, and the oxygen atoms plus Van der Waals radius of the oxygen atom. Figures in brackets indicate the dimensions of ions or polar groups, calculated from available data on interatomic distances (Sutton, 1965) including Van der Waals radius; r_1 applies to solid angles: it represents the length of the arm extending from the center of symmetry with respect to *charge* distribution; r_2 is the radius from the center of symmetry (planar) in a triangle with equal charges at each corner; r_3 the length of the arm C–O in COO^- (plane angle). ClO_4^- is a regular pyramid with chloride at the center and four negative charges evenly distributed at the corners. The shape of BrO_3^- and ClO_3^- is also pyramidal, but the halide (bromide or chloride) is charge-free. NO_3^- and COO^- are planar; the charge is evenly distributed among oxygen atoms. SCN^- is linear (total length, including Van der Waals radii = 6.13 Å; the charge is distributed between N^- and S^-; Van der Waals radii at each end are 1.50 and 1.85 Å, respectively). Hydration energy and relative hydrated size are taken from Araki *et al.* (1961); limiting equivalent conductivities from Robinson and Stokes (1970).

The constant K^A_{Cl}, as well as ion permeability, do not depend on naked ion size of hydrated size (Eisenman, 1965; Diamond & Wright, 1969); the figures given in Table 1, column 3, and Table 2, columns 1 and 2 are

consistent with this statement. The affinity constant K_{Cl}^{A} is a function of the difference in partial molal free energies of hydration of Cl^- and the anion A^- on the one hand and the difference of free energies of interaction of these anions with the membrane on the other hand (Eisenman, 1965). If it is assumed that the differences in hydration energy (Table 2, column 3) represent a good approximation of the differences in partial molal free energies of hydration (that is the differences of entropy of hydration are neglected), it becomes obvious that hydration energy is not critical in the determination of K_{Cl}^{A}; the hydration energies of ClO_4^- and of I^- are similar to those of NO_3^- and SCN^-, when the respective K_{Cl}^{A}'s differ considerably. Thus, high K_{Cl}^{A}'s at the shunt pathway of *Necturus* should be primarily ascribed to high affinity of the sites for ClO_4^- and I^-. This is not surprising, since electrostatic forces are heavily involved in ion-site interactions; ClO_4^- is the least hydrated among nonorganic anions and is likely to be most attracted by weak sites. A thorough understanding of such interactions would require detailed information on the chemistry of the sites.

One may speculate, however, on their properties by comparing the K_{Cl}^{A}'s to the structure of interacting anions. Table 2, column 1 indicates the size of naked ions and polar groups used in this study, i.e. the space presumably filled by the interacting anion or polar group, stripped from its hydration shell, when it binds (if it does) to the positive site. ClO_4^- and I^-, the ions with the highest K_{Cl}^{A}'s, are the two largest among anions displaying perfect spherical symmetry with respect to charge distribution ($ClO_4^- > I^- > Br^- > Cl^- > F^-$, Table 2). By comparison, the binding constant of anions or polar groups of similar size to ClO_4^- and I^- (i.e. BrO_3^-, SO_3^-, COO^-) is consistently smaller. It is suggested that the structure of the sites is suited to accommodate large anions in which charge distribution complies with spherical symmetry; the slow reversibility of the bonds "site-ClO_4" and "site-I" supports this view. From the size of ClO_4^-, the anion displaying the highest binding constant, the radius of the interacting sites may be tentatively estimated close to, or slightly greater than 2.90 Å.

In the foregoing it was largely assumed that anion permeation occurs via a positively charged pathway. However, there is no theoretical reason to rule out, *a priori*, a concomitant hinderance of physiologic ion permeation, especially chloride permeation. This possibility does not invalidate the fixed positive charge theory. It does modify, however, the quantitative analysis, since part of the decrease in transepithelial conductance, g_A, could be ascribed to a decreased chloride mobility; the use of Eq. (4) would underestimate u_A/u_{Cl}. Eqs. (5) and (6) (*see* Appendix) appear

more appropriate to describe anion permeation. Unfortunately they contain too many unknowns to be of any practical use in this study. They do extend, however, the validity of the theory of anion permeation through positively charged pores to those observations in which hinderance of physiologic ion permeation may also occur.

It is highly unlikely that such a mechanism is primarily involved during ClO_4^- and I^- substitution, since it cannot account for the incomplete reversibility of conductance changes (irreversible damage of the shunt pathway by these ions should also alter the amplitude of bi-ionic potentials in subsequent determinations; this was not observed). It may account for the variability of the computed u_A/u_{Cl}'s and K_{Cl}^A's among anions having the same polar group (COO^-) although other factors may also be responsible for such variability (decreasing mobility with increasing size; electrostatic forces between membrane sites and parts of the molecule other than the polar end COO^-; poor determination of u_A/u_{Cl} and K_{Cl}^A). To what extent some foreign anions may hinder chloride permeation through the shunt pathway cannot be assessed from the present work. The basic conclusions would not be affected though, since this hypothesis does not appear as an effective challenge to the concept of anion permeation through positive fixed charges.

Appendix

In membranes in which anion permeation occurs through fixed positively charged pores, the permeability ratio P_A/P_{Cl} is given by (Conti & Eisenman, 1965; Hagiwara *et al.*, 1971):

$$P_A/P_{Cl} = (u_A/u_{Cl})\, K_{Cl}^A \tag{3}$$

where u_A/u_{Cl} is the mobility ratio within the membrane and K_{Cl}^A the partition coefficient or binding constant of the membrane for the ions A^- and Cl^-. The conductance ratio g_A/g_{Cl} in an anion-selective membrane is given by (Conti & Eisenman, 1965; Hagiwara *et al.*, 1971):

$$\frac{g_A}{g_{Cl}} = \frac{\ln(u_{Cl}/u_A)}{(u_{Cl}/u_A) - 1}. \tag{4}$$

Eq. (4) was developed on the assumption of no cation conductance and of constant mobilities within the membrane for the anion species A^- and Cl^-. If the foreign anion interferes with membrane structure and

shifts the value of chloride mobility from u_{Cl} to u'_{Cl} ($u'_{Cl} < u_{Cl}$), Eq. (4) becomes unsuitable to estimate u_A/u_{Cl} from g_A/g_{Cl}. The relation between conductances and mobilities is given (from Eqs. 5 and 6 in Hagiwara *et al.*, 1971, by substituting u'_{Cl} for u_{Cl} when necessary) by:

$$\frac{g_A}{g_{Cl}} = \frac{u'_{Cl}}{u_{Cl}} \frac{\ln(u'_{Cl}/u_A)}{(u'_{Cl}/u_A) - 1}. \tag{5}$$

Eq. (4), which provides the ratio of anionic permeabilities, should be corrected accordingly:

$$P_A/P_{Cl} = (u_A/u'_{Cl})\, K^A_{Cl} \tag{6}$$

unless u_A and u_{Cl} undergo proportional changes as a function of A_0.

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