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pH-Dependent Electrical Properties and Buffer Permeability of the *Necturus* Renal Proximal Tubule Cell

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Summary. *Necturus* kidneys were perfused with Tris-buffered solutions at three different pH values, i.e. 7.5, 6.0 and 9.0. A significant drop in fluid absorption occurred at pH 6.0, whereas pH 9.0 did not increase volume flow significantly. When acute unilateral, i.e. either in the lumen or the peritubular capillaries, and bilateral pH changes were elicited in both directions from 7.5 to 9.0 at a constant Tris-butyrate buffer concentration, both peritubular membrane potential difference V_1 and transepithelial potential difference V_3 hyperpolarized, independently of the side where the change in pH was brought about. Acid perfusions at pH 6.0 caused a similar response but of opposite sign. Analysis of the potential changes shows that pH influences not only the electromotive force and resistance of the homolateral membrane, but also the electrical properties of the paracellular path. Interference of pH with Na, Cl or K conductance was assessed. Any appreciable role for sodium or chloride was excluded, whereas the potassium transference number (t_K) of the peritubular membrane increased 16% in alkaline pH. However, this increase accounts only for 19 to 36% of the observed hyperpolarization. Since changes in Tris-butyrate buffer concentration at constant pH do not affect V_1 or V_3 considerably, the hyperpolarization in pH 9 cannot be explained by an elevation in internal pH only, or by a Tris-H^+ ion diffusion potential only. The role of the permeability of the buffers: bicarbonate, butyrate and phosphate, in determining electrical membrane parameters was evaluated. Transport numbers of the buffer anions ranked as follows: $t_{HCO_3} > t_{butyrate} > t_{phosphate}$. It is concluded that modulation of membrane potential by extracellular pH is mediated primarily by a change in peritubular cell membrane t_K and additionally by membrane currents carried by buffer anions.

Key Words pH · K channels · buffer permeability · organic anion transport · electrical properties · transference numbers · *Necturus* · proximal tubule

Introduction

According to the fixed-charge theory [80] and the equilibrium ion specificity theory [29], the molecular structures responsible for ion discrimination across a membrane can be charged sites such as carboxyl, phosphate or amino groups or induced dipoles of which the field strength determines the permselectivity. Coulombic forces could control cationic versus anionic permeability and discriminate between ions of the same sign. The effective charge of membrane sites could be changed by protonation depending on their pK_a and the ambient pH. Therefore pH alterations of the medium could shift the cation versus anion selectivity of the membrane, or change the permeability sequence of the alkali cations, if this sequence is mainly due to equilibrium selectivity rather than to mobility differences [29]. The permeability to cations and anions is a function of the ambient pH in a variety of membranes: in artificial weak-acid or weak-base ion-exchanger membranes [41], in erythrocytes [60, 62], yeast cells [4], barnacle muscle [40], frog skeletal muscle [44], crayfish giant axon [79], myelinated nerve [42], Purkinje fibers [22], rat liver [24], small intestine [70], choroid plexus [88], gallbladder [58], turtle bladder [77], toad bladder [53], frog skin [86], cornea [30, 45], retinal pigment epithelium [46] and in kidney proximal tubule [2, 5, 32–34, 76, 81].

Several observed effects of pH changes on renal tubules could be related to an altered permselectivity of a membrane barrier. In the rat proximal tubule the fluid reabsorptive rate is unaffected when the pH of the solution infused into the capillaries is changed at constant HCO_3 concentration from 6.95 to 8.1 [85]. In contrast, in rabbit isolated proximal convoluted tubules both low and high pH of the bath medium reduce the rate of fluid absorption [25]. In *Necturus* proximal tubule a perfusion of the kidney with a solution of normal bicarbonate but high CO_2 content at pH 6.8 depresses sodium and fluid absorption [87]. Mammalian proximal tubule studies suggest that the presence of bicarbonate or certain other buffers is essential for fluid and salt absorption [54, 84, 85]. Since in *Necturus* proximal

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tubule a peritubular and luminal change in bicarbonate concentration does not affect fluid reabsorption, whereas a fall in pH to 6.8 does [87], in the present study the pH effect on fluid absorption in *Necturus* is investigated using Tris as buffer instead of bicarbonate.

In the present experiments we investigate how extracellular pH controls the electrical properties of the peritubular cell membrane in *Necturus* proximal tubule. However, any effect of extracellular pH changes may be mediated by secondary effects such as intracellular pH changes and intra- or extracellular concentration changes of permeable buffer species. Bicarbonate buffer causes a decrease in the cellular pH and in the passive membrane permeability to ^{42}K in rabbit renal cortex slices when incubated at pH 6.2, whereas intracellular pH remains high when a Tris-TES buffer is used [59]. The pH dependence of the peritubular cell membrane potential difference in *Necturus* proximal tubule is reported to vary with the nature of the buffer species present [81]. Therefore, in the present experiments we used different buffers at constant pH in order to determine the role of buffer permeation in the electrical behavior of the proximal tubule cell membrane.

Materials and Methods

Animal Preparation

Adult *Necturi* of both sexes were obtained from Mogul-Ed Company (Oshkosh, Wisconsin), stored in charcoal-filtered water at 15°C and fed live goldfish. Anesthesia was induced by immersion in 660 mg/liter tricainemethane sulfonate (Finquel) and maintained by including 66 mg/liter tricaine in the vascular perfusion solutions. The animals were placed supine on a specially designed Lucite® table as described by Spring and Paganelli [74].

In order to prevent spontaneous muscle contractions in the course of the experiment, the animals were pithed following transection of the tail and in addition the spinal cord was depolarized by injection of 3 M KCl into the vertebral canal with a long needle (gauge 30). This procedure prevented all movements afterwards.

In order to allow fast perfusion changes of the kidneys the surgical procedure was as follows. A paramedian abdominal incision was made from the cloaca to the thoracic region. The iliac arteries and veins were ligated. The urinary bladder and lower segments of the intestine were removed after cauterization of the mesenteric vasculature. The spermatic or ovarian blood vessels were also cauterized and the genital organs removed so that both kidneys were completely exposed for micropuncture. The aortic perfusion was initiated by cannulating the dorsal aorta in the mid-thoracic region with polyethylene tubing (PE 90). An outflow catheter (PE 240) was inserted into the postcaval vein and provided drainage with an opening at the level of the kidney surface. The aortic and caval catheters were advanced caudally until they reached the transition between the genital and the pelvic portion of the kidneys. A ligature cephalad to the catheter tips prevented leakage from the postcardinal veins. In the experiments where the effect of sudden changes of pH was studied, a polyethylene catheter (PE 20) was inserted into the renal portal vein of one kidney. In order to produce fast changes in the perfusion fluid of the peritubular capillaries a needle (gauge 30) connected to the substitution fluid was placed inside the PE 20 catheter such that the orifice emerged at the tip of the catheter in the portal vein, thereby reducing dead space [72]. In the steady-state experiments following transection of the tail the caudal vein was catheterized with a PE 60 tubing for perfusion of the protal vein of both kidneys. This procedure effected a separate perfusion of the glomeruli and renal portal system. Figure 1 illustrates the changes in peritubular capillary composition (*p*).

Perfusion rates were controlled and adjusted throughout the experiment with the aid of rotameter-type flowmeters (Brooks Instruments Co. No. 1-15-6) at a steady level of 1.5, 0.5 and 1 ml/min through the aorta, renal portal vein and caudal vein, respectively. Homogeneous distribution of the perfusate throughout the kidney was checked visually during the experiments by single injections of small amounts of 0.05% F.D.C. green Ringer's solution (Keystone Aniline and Chemical Co., Chicago, Ill.). Solutions of similar composition to the portal perfusion superfused the kidney surface with a device allowing rapid switches between solutions of different composition (Fig. 1, *p*.). The kidney surface was illuminated by a fiber optic light pipe with a tungsten halide light source (Fiber-Lite). Micropuncture was carried out with the aid of a stereomicroscope (Leitz) under 10, 40 or 100 magnification. A rapid unilateral luminal microperfusion change was obtained by means of a double-barrelled micropipette as shown in Fig. 1(*l*). These micropipettes were made out of borosilicate glass capillaries with 0.8 mm o.d. (Corning 7740). Both barrels were joined together with high Pyseal® cement (Fisher Scientific Company). The tips were doubly ground and had a diameter of about 15 μm. The nephra were punctured either in the glomerulus or just after the branching of the nephrostome. Sudden changes of the peritubular perfusion fluid were obtained by changing simultaneously the perfusion fluid above the kidney surface and the perfusion of the renal portal vein. The arrangement of Fig. 1 allowed fast unilateral and bilateral perfusion changes along the tubule epithelium.

Net Volume Flux Measurements

Fluid reabsorption by the straight segment of the late proximal tubule was measured using the stationary microperfusion technique, adapted for the *Necturus* [14, 36]. The method used for determination of volume flux is that described by Forster, Steels and Boulpaep [31]. Tubules were studied in steady state in kidneys doubly perfused with solutions at pH 7.5, 6.0 or 9.0. In contrast to preparations used for electrical measurements, 15 g/liter polyvinylpyrrolidone [average molecular weight 40,000 (Plasdone C, GAF Corp., Dyestuff and Chemical Div., New York)] was added to the superfusion fluid to insure that the bathing fluid of the kidney had a similar colloid osmotic pressure as the vascular fluid.

Electrical Measurements

Ling-Gerard microelectrodes, pulled from borosilicate glass capillary tubing (Corning No. 7740, o.d. 0.9 or 1 mm, i.d. = 0.6 mm), were filled with 1.8 M K-citrate and 0.8 M KCl, titrated to pH 7.0 by means of 1 M citric acid, in an attempt to obtain low tip

Table 1. Composition of solutions (in mM)[a]

	Tris Control pH 7.5	Tris pH 9	Tris pH 6.0	Low Na pH 7.5	Low Na pH 9.0	Low Cl pH 7.5	Low Cl pH 9.0	High K pH 7.5	High K pH 9.0	Tris 40 mM	Bicarbonate 25 mM	Butyrate 28 mM	Phosphate 22 mM
	a)	b)	c)	d)	e)	f)	g)	h)	i)	j)	k)	l)	m)
pH	7.5	9.0	6.0	7.5	9.0	7.5	9.0	7.5	9.0	7.5	7.5	7.5	7.5
Na^+	95.05	97.85	95.0	5.05	7.85	95.05	97.85	77.55	80.35	59.05	103.05	103.05	119.05
K^+	2.5	2.5	2.5	2.5	2.5	2.5	2.5	20	20	2.5	2.5	2.5	2.5
Ca^{2+}	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	0.5
Mg^{2+}	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Tris-H^+	3.2	0.4	4.0	3.2	0.4	3.2	0.4	3.2	0.4	32	—	—	—
Tris	0.8	3.6	—	0.8	3.6	0.8	3.6	0.8	3.6	8	—	—	—
L-Lysine-H^+	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Tetramethylammonium	—	—	—	95	95	—	—	—	—	—	—	—	—
Cl^-	99.7	99.7	100.7	104.7	104.7	0.2	0.2	99.7	99.7	92.5	79.7	79.7	79.7
Lactate$^-$	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	1.0
D-L-Glutamate$^-$	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Butyrate$^-$	3.2	3.2	3.0	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.0	28.0	3.0
Isethionate$^-$	—	—	—	—	—	95	95	—	—	—	—	—	—
SO_4^{2-}	—	—	—	—	—	2.25	2.25	—	—	—	—	—	—
HCO_3^-	—	—	—	—	—	—	—	—	—	—	25	—	—
$H_2PO_4^-$	—	—	—	—	—	—	—	—	—	—	—	—	3.0
HPO_4^{2-}	—	—	—	—	—	—	—	—	—	—	—	—	19.0
D-Glucose	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22
D-L-Alamine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Glutamine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sucrose	—	—	—	—	—	—	—	—	—	—	—	—	—

[a] All vascular perfusion polutions contained in addition: Polyvinylpyrrolidone, GAF Corporation, Plasdone C, average molecular weight 40,000, 15 gram/liter; Heparin, 2,000 units U.S.P.; Tricaine methane sulfonate 66 mg/liter, originating from stock solutions at neutral pH. All solutions were equilibrated with 100% O_2, except for solution K (10 mM HCO_3^-) 98.5% CO_2 and 1.5% CO_2; for solution *p* (25 mM HCO_3^-) 96% O_2 and 4% CO_2.

potentials [64]. Filling procedure and connections of microelectrodes, as well as the arrangement of the reference electrode and the potential difference recording equipment were described earlier [31]. A correction for the microelectrode tip potential change due to a change in composition of the perfusion solution was determined in vitro for each microelectrode after its use and withdrawal from the tubule lumen. A broken microelectrode inserted in a Lucite holder with a sidearm for pressure application created a flowing junction at the broken tip serving as a reference electrode. The asymmetry due to the tip potential of the microelectrode was checked immediately after its use by breaking off the tip in the bath above the kidney surface. Tip resistance between 10 and 40 MΩ and tip potentials less than 5 mV were criteria for reliable microelectrodes. The peritubular cell membrane potential difference V_1 was defined as the potential difference between the reference electrode in the bath and the probing microelectrode when impaling the cell interior. The transepithelial potential difference V_3 was recorded when the same probing microelectrode was further advanced into the lumen (Fig. 1). The criteria for microelectrode tip localization and stability of recording were defined earlier [15]. Electrical measurements were mostly performed on the early segments of proximal tubules.

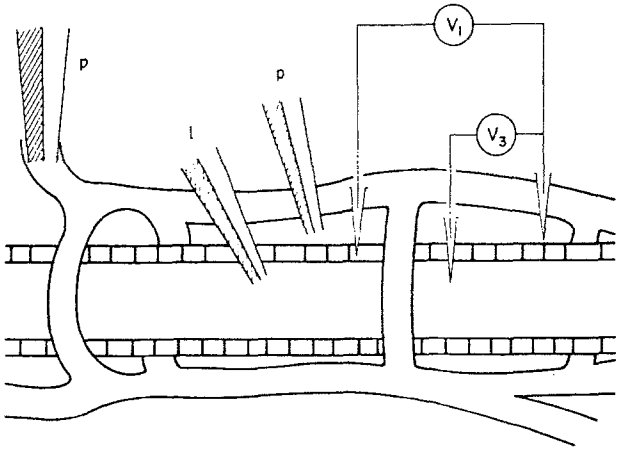

Fig. 1. Methods used for electrical measurements of basolateral membrane potential difference (V_1) and transepithelial potential difference (V_3), while the composition of luminal (*l*) and peritubular (*p*) perfusion solutions could be changed separately or simultaneously

Solutions

Table 1 lists the composition of the solutions. The effect of acute pH changes was investigated using solutions buffered with Tris-butyrate. Other buffer species such as lactate and butyrate were present in all solutions, since these solutes together with other neutral substrates are essential for maintaining optimal transport function by the proximal tubule [31]. The pH 7.5 control solution (Table 1,a) was titrated with NaOH to obtain pH 9 (solution b) or with HCl to obtain pH 6 (solution c). In the low-sodium solution (d) 95 meq tetramethylammonium chloride substituted for NaCl. Since this solution was titrated with NaOH to pH 9.0 to form solution (e) about the same amount of NaCl was added to solution (d) at pH 7.5. In the low-chloride solution (f) at pH 7.5 the following substitutions were done: NaCl by Na-isethionate, KCl by K_2SO_4 and $MgCl_2$ by $MgSO_4$. Low-chloride solution (g) at pH 9 was obtained by NaOH titration of solution (f). In the high-K solution (h) 20 mM KCl substituted for NaCl and a similar high-K pH 9 solution (i) was obtained by NaOH titration of solution (h). In the high-buffer concentration solution (j) 36 meq NaCl was

Table 2. Effect of changes of pH on fluid reabsorption

			Luminal diameter 10^{-4} cm	Rate constant 10^{-3} min^{-1}	H_2O flux (J_v) 10^{-9} liter cm^{-2} sec^{-1}
a)	pH 7.5	Tris buffer	74.0 ± 2.49 (15)	−14.2 ± 1.30 (15)	−0.43 ± 0.32 (15)
b)	pH 9.0	Tris buffer	75.2 ± 3.35 (11)	−17.0 ± 3.46 (11)	−0.53 ± 0.115 (11)
c)	pH 6.0	Tris buffer	71.9 ± 2.34 (8)	−3.9 ± 2.07 (8)[a,b]	−0.11 ± 0.058 (8)[a,b]

[a] Value significantly different from the corresponding in pH 9.0, $P < 0.01$.
[b] Value significantly different from the corresponding in pH 7.5, $P < 0.01$.

Table 3. Effects of changes of pH at constant buffer concentration

Perfusion solution	pH 7.5[a]	pH 7.5[a]
V_1^c	−45.1 ± 0.8 (147)	−47.7 ± 1.8 (83)
V_3^c	−5.6 ± 0.8 (43)	−7.7 ± 0.6 (72)
Perfusion solution	**pH 9.0[a]**	**pH 6.0[a]**
ΔV_1^p "on"[b]	−10.8 ± 1.7 (18)[c]	+11.2 ± 1.0 (48)[c]
ΔV_1^p "off"[b]	+7.2 ± 1.8 (13)[c,d]	−6.2 ± 0.8 (32)[c,d]
ΔV_3^p "on"	−0.3 ± 0.2 (5)	+1.0 ± 0.3 (44)[c]
ΔV_3^p "off"	0 ± 0 (4)	−0.5 ± 0.2 (44)[c,d]
ΔV_1^l "on"	−5.5 ± 0.4 (56)[c]	+9.2 ± 0.9 (29)[c]
ΔV_1^l "off"	+3.6 ± 0.4 (47)[c,d]	−8.1 ± 1.3 (16)[c]
ΔV_3^l "on"	−0.8 ± 0.2 (44)[c]	+1.0 ± 0.3 (28)[c]
ΔV_3^l "off"	+0.9 ± 0.2 (39)[c]	−0.8 ± 0.5 (23)
ΔV_1^{pl} "on"	−7.4 ± 0.6 (42)[c]	+12.4 ± 5.9 (4)[c]
ΔV_1^{pl} "off"	+5.0 ± 0.7 (33)[c,d]	−10.7 ± 5.8 (3)
ΔV_3^{pl} "on"	−0.1 ± 0.3 (14)	+1.3 ± 0.9 (3)
ΔV_3^{pl} "off"	−0.2 ± 0.3 (11)	−1.0 ± 2.0 (2)

[a] pH 7.5 is solution a, pH 9.0 is solution b, and pH 6.0 is solution c of Table 1.
[b] "On" indicates the response of the potential difference at the initiation of pH 9.0 or 6.0, whereas "off" indicates the response during a return to pH 7.5.
[c] Significant change in potential difference $P < 0.01$.
[d] Indicates that the absolute value of the "off" response is significantly different from the "on" response in a paired comparison, $P < 0.05$.

replaced by Tris-Cl at pH 7.5. High concentrations of weak-acid buffers were prepared by replacing an adequate amount of chloride by the following anion buffers: bicarbonate (k), butyrate (l) and phosphate (m) at pH 7.5. All experimental solutions contained 0.05% FDC green in order to visually check the adequacy of the perfusion change.

The data are expressed as means ± standard error of the mean. Statistical significance between groups was calculated by means of a Student's *t*-test for unpaired comparisons.

Results

Effect of pH Changes and Buffer Type on Fluid Reabsorption

The effect of long-term changes of pH on fluid reabsorption are summarized in Table 2. At pH 7.5 volume reabsorption J_v amounted to -0.43×10^{-9} liter cm^{-2} sec^{-1}. A significant drop to -0.11×10^{-9} liter cm^{-2} sec^{-1} occurs at pH 6, whereas pH 9 does not increase the volume flow significantly. These results confirm the observation by Whittembury et al. [87], indicating that pH 6.8 with HCO_3 buffer diminishes the volume flux to 21% of the control value in *Necturus* proximal tubule. A similar observation was made by Maude [56] between pH 7.0 and 7.8 in rat kidney. In contrast, a change in pH from 6.9 to 8.1 with bicarbonate buffer or from 7.5 to 8.1 with glycodiazine buffer has an insignificant influence on the reabsorptive rate in other rat studies [85].

The control J_v in pH 7.5 Tris-butyrate buffer is significantly less than the value of -0.73×10^{-9} liter cm^{-2} sec^{-1} observed in *Necturus* kidney with bicarbonate buffer at pH 7.5 [31] and of -0.83×10^{-9} liter cm^{-2} sec^{-1} at pH 7.6 [61]. These data confirm reports in mammalian proximal tubule that the nature or permeability of the buffer may be critical for fluid absorption [20, 37, 52, 54, 56, 67, 84, 85]. Thus, it is important to explore both the influence of pH and buffer species on membrane transport parameters.

Effect of pH Changes at Constant Buffer Concentration on Electrical Parameters

The effects of acute unilateral and bilateral pH changes in both directions from 7.5 to 9.0 or from 7.5 to 6.0 at a constant buffer concentration of 4 mM Tris and 3.2 mM butyrate were measured on the potential differences of the *Necturus* proximal tubule. Because these experiments were performed during the summer months from June until August the control peritubular membrane potential differences at pH 7.5 (V_1^c) are rather low, −45 to −48 mV in the two control groups (Table 3). Transepithelial potential difference at pH 7.5 (V_3^c) ranges between −0.5 and −13 mV with a mean value of −5.6 and −7.7 mV for the control group of the alkaline and acid perfusion, respectively (Table 3).

Since the luminal and peritubular membrane of the proximal tubule are asymmetric and electrically coupled through the presence of an intercellular

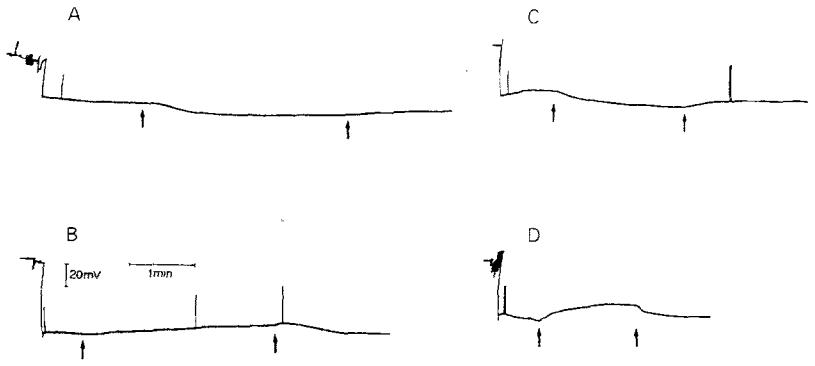

Fig. 2. Typical recordings of V_1 when the peritubular solutions were changed from pH 7.5 to pH 9 (*A*) and 6 (*B*) and back to 7.5. The arrows mark the moments at which the solutions were changed. (*C*) and (*D*) represent recordings when the luminal microperfusion solutions were changed to pH 9 and 6, respectively

shunt pathway [12], the effects of pH alterations were investigated on both membranes separately and simultaneously. Thus, changes in peritubular cell membrane potential difference (ΔV_1) and transepithelial potential difference (ΔV_3) were monitored during pH alterations either single sided in the peritubular capillaries and superfusion (superscript *p*) or single sided in the lumen (superscript *l*), or bilaterally (superscript *pl*). Changes in potential difference for the "on" response are defined as experimental-control, i.e. for the peritubular membrane potential $\Delta V_1^p = V_1^p - V_1^c$; $\Delta V_1^l = V_1^l - V_1^c$; $\Delta V_1^{pl} = V_1^{pl} - V_1^c$, and for transepithelial potential difference $\Delta V_3^p = V_3^p - V_3^c$; $\Delta V_3^l = V_3^l - V_3^c$; $\Delta V_3^{pl} = V_3^{pl} - V_3^c$. The "off" response has the opposite sign by definition. In order to prevent alteration of the composition of the luminal perfusion fluid due to leakage from the peritubular perfusate with a different composition, the tubule lumen was perfused with a micropipette at a sufficiently high rate to maintain luminal fluid composition constant. Peritubular and luminal perfusions were alternated in a random sequence.

As can be seen in Fig. 2 the response of the peritubular membrane potential difference ΔV_1^p and ΔV_1^l to pH alterations is relatively prompt. The half-time of the "on" response to peritubular pH changes (Fig. 2*A* and *B*) carefully measured in 38 cases, is 57.2 ± 5.7 sec (n = 38). For luminal pH changes (Fig. 2*C* and *D*) the half-time of the initial "on" response is 14.9 ± 1.3 sec (n = 28). As shown in Fig. 2, the half-time of the "off" response does not differ significantly from the "on" response. During the peritubular alterations the tubule epithelium was exposed from 70 to 640 sec to the different pH's and only a single time constant was observed. During the luminal pH changes exposure lasted from 30 to 190 sec and usually two time constants were observed.

A sudden switch to the alkaline perfusion solution of pH 9 hyperpolarizes both V_1 and V_3, independently of the side where the change in pH is brought about: peritubular alkalinization hyperpolarizes V_1 by −10.8 mV and V_3 by −0.3 mV; luminal alkalinization hyperpolarizes by −5.5 and −0.8 mV, respectively; and bilateral alkalinization hyperpolarizes by −7.4 and −0.1 mV, respectively (Table 3). The same behavior but of opposite sign is observed after perfusion with solutions of pH 6.0. Peritubular acidification induces a steady-state depolarization of V_1 and V_3 of +11.2 mV and +1.0 mV, respectively; luminal acidification leads to a depolarization of +9.2 and +1 mV, respectively; and bilateral acidification elicits depolarizations of +12.4 and +1.3 mV, respectively. The steady-state changes in potential difference are listed in Table 3.

The changes in potential difference due to pH alterations are not completely reversible. The amplitude of the "on" response at the initiation of pH 9.0 is significantly higher than during the return of pH 7.5 ("off" response) for the peritubular membrane potential differences in the three different perfusion situations: ΔV_1^p, ΔV_1^l, ΔV_1^{pl}. In contrast, among the responses to acidification, only the recovery ΔV_1^p of the peritubular membrane potential difference and the recovery ΔV_3^p of the transepithelial potential after peritubular acidification remain incomplete (Table 3).

Ionic Basis of the pH-Induced Changes in Electrical Parameters

The pH effects on the electrical potential differences could be due to alterations of a number of determinants of membrane potential. Firstly, changes in protonation of specific sites of ion channels for, e.g., Na, Cl, K could affect their channel conductance. Secondly, changes in the extra- or intracellular chemical activity of protons, of charged buffer species or other ions could generate ion diffusion potentials across one or more intraepithelial barriers. In view of the low proton activity as compared to that of other ions, the transference number for hydrogen ions is expected to be negligible and therefore will not appreciably influence the membrane potential. Thirdly, the changes in electrical potential difference could result from alterations in electrogenic transport systems which respond to

Table 4. Peritubular membrane hyperpolarization at pH 9.0: Role of Na or Cl

Perfusion solution (in meq)	Na^+ 95, Cl^- 100[a]	Na^+ 5, Cl^- 105[a]	Na^+ 95, Cl^- 0.2[a]
V_l^c	−65.9 ± 2.2 (17)	−45.2 ± 1.3 (51)[b]	−68.6 ± 1.0 (76)
ΔV_l^p "on"[c]	−6.0 ± 0.9 (14)[d]	−8.1 ± 0.7 (40)[d]	−5.8 ± 0.7 (41)[d]
ΔV_l^p "off"	+5.2 ± 1.0 (12)[d]	+7.5 ± 0.4 (29)[d]	+4.1 ± 0.7 (26)[d]

[a] Control solutions are solutions a and b, low Na solutions are d and e, low Cl solutions are f and g of Table 1.
[b] Indicates a value significantly different from control perfusion with Na^+ 35 and Cl^- 100 meq.
[c] "On" indicates the change in peritubular membrane potential at the initiation of pH 9.0, whereas "off" indicates the response during a return to pH 7.5.
[d] Significant change in potential difference $P < 0.001$.

changes in extracellular or intracellular pH, such as symporters [1, 11, 39, 89], or pumps [9, 26].

In view of the sizeable effect of pH on the peritubular membrane potential difference we further investigated as a typical experimental maneuver, the effect of a peritubular pH change from 7.5 to 9.0 on the peritubular membrane potential difference. Interference of pH with Na, Cl or K channels was assessed. The contribution of sodium and chloride was determined in experiments in which sodium or chloride was omitted from the solution. Table 4 summarizes the absolute values and changes in peritubular potential differences during these different conditions. The mean control value of −66 mV is markedly higher than the value of −45 mV observed in Table 3. However, the experiments of Table 4 were carried out on winter animals. Seasonal variations in control values of fluid absorption, sodium flux, cell composition and peritubular membrane potential differences have been well documented [14, 71]. In these winter animals peritubular alkalinization hyperpolarizes the peritubular membrane by −6 mV. The return to pH 7.5 is attended by an almost complete recovery of the potential difference.

In order to examine the role of Na in the alkalinization response we perfused the kidney with a solution containing only 5 meq sodium and 95 meq tetramethylammonium (TMA). Replacement of sodium with TMA or other substitutes causes the cellular sodium concentration and activity to fall together with the net sodium flux [49, 65, 71, 83]. In the present experiments (Table 4) substitution of sodium by TMA causes a fall in peritubular membrane potential difference to −45.2 mV, similar to earlier findings [11, 49, 71, 83]. The kidneys were equilibrated for about one hour with the low-sodium perfusion before potential differences were measured. Switching to the alkaline solution causes also a hyperpolarization of −8.1 mV, which is practically completely reversible and neither significantly different from that observed in the winter animals perfused with a normal NaCl Ringer's (−6 mV) (Table 4) nor from the value (−10.8 mV) observed in summer animals with a low peritubular membrane potential difference (Table 3). These observations argue against the electrical response to pH to be caused by a pH dependence of Na channels, of Na-linked rheogenic transporters or of the sodium-potassium ATPase.

The hyperpolarization due to the alkaline medium could also be explained by a decrease in anion permeability [17]. Intracellular chloride activity in amphibian proximal tubules [11, 27, 35, 38, 48, 73] exceeds that required for electrochemical equilibrium at the actually measured membrane potential difference (V_l). A sizeable chloride conductance across the peritubular membrane was found using sulfate replacements and low calcium [12], whereas cell membrane conductance did not change sizeably when gluconate or other organic anions substitute for chloride [3, 38]. In the present experiments the kidney was equilibrated with isethionate Ringer's (solution f, Table 1) for about one hour before potential measurements were started. At that time chloride concentration measured in the effluent of the postcaval vein amounts to only about 3 meq. At the same time intracellular chloride concentration should be low [38, 73]. With both intra- and extracellular reduced chloride activity, a decrease in chloride membrane conductance caused by pH 9 would have a very small effect on the cell potential difference. However, as shown in Table 4 a hyperpolarization ΔV_l^p of −5.8 mV is measured, equivalent to the value in control conditions. These observations exclude any appreciable role for chloride in the mechanism of hyperpolarization of V_l due to pH 9. It is noteworthy that the peritubular potential difference of pH 7.5 during isethionate perfusion is not different from the control value during Cl Ringer's perfusion. The same steady-state insensitivity of this potential difference to Cl^- concentration was

observed [73] when Na_2SO_4 Ringer's substituted for NaCl Ringer's. This confirms that chloride traverses the peritubular membrane in an electroneutral way [38].

In order to investigate the role of K in the hyperpolarization due to alkaline pH, alterations in relative potassium conductance were examined. The relative contribution of potassium to the membrane potential difference or K transference number can be determined by a rapid displacement of the K chemical potential as described for single striated muscle fibers [43], crayfish giant axons [78], and applied to *Necturus* kidney tubule [12, 13]. The effect of a sudden step of the extracellular potassium concentration from 2.5 to 20 mM at constant Cl^- concentration on the membrane potential difference was determined and compared in the same kidney when perfused with Tris-Ringer's solution at pH 7.5 and when perfused at pH 9.0. The amplitude of the potassium-induced depolarization was determined at both pH values in either the same or different tubule cells. The absolute value of V_l for tubule cells exposed to pH 7.5 was -61.1 ± 2.0 mV ($n = 26$), as compared to -67.8 ± 1.9 mV ($n = 30$) for the pH 9 perfusion, which is a significant increase ($P < 0.025$). The magnitude of the depolarization elicited by 20 mM K as represented in Fig. 3, is $+29.2 \pm 1.7$ mV ($n = 16$) at pH 7.5 versus $+33.8 \pm 1.2$ mV ($n = 22$) at pH 9, which is significantly different ($P < 0.05$). These data allow a calculation of the apparent transference number for K according to

$$t_K = \frac{\delta V_l}{25.25\delta \ln[K]_o} \quad (1)$$

at constant intracellular K concentration. The calculated transference number of potassium is 0.56 at pH 7.5 and 0.64 at pH 9, an increase of 16%. This observation is qualitatively similar to the effect of pH on ion selectivity in giant axons [21, 79], in giant barnacle muscle [40] and in liver cells [24].

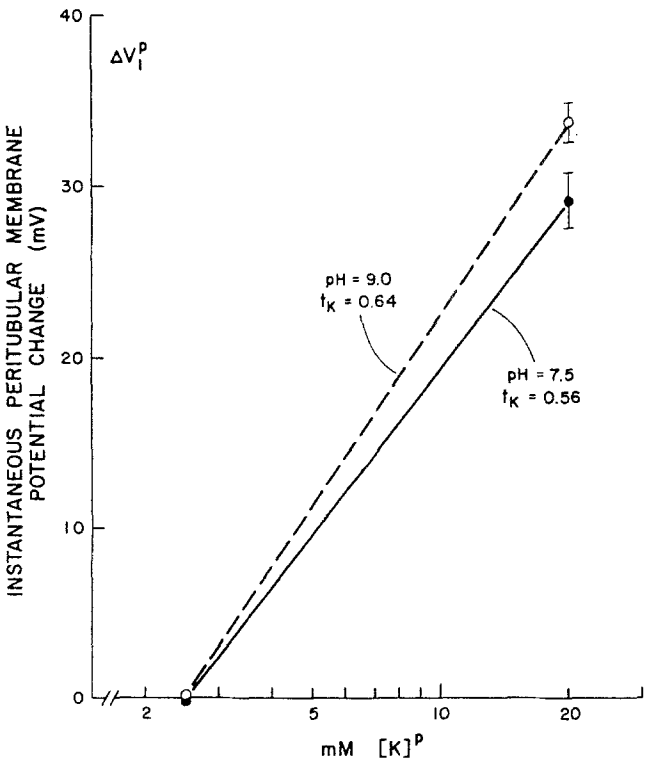

Fig. 3. Instantaneous peritubular membrane potential change (ΔV_l^p) when the peritubular potassium concentration was increased from 2.5 to 20 mM at pH 7.5 and 9

Effect of Extracellular Buffer Concentration at Constant pH on Electrical Parameters

Since changes in extracellular pH at constant total buffer concentration are accompanied by changes in the ratio of ionic to nonionic forms of weak acids and bases in the solution, changes in extracellular concentration of these charged versus uncharged species may be responsible for the pH-dependent effects. Thus, changes in the diffusion potential of charged buffer species or changes in intracellular pH (pH_i) due to nonionic diffusion may result. Titration of a Ringer's solution between pH 7.5 and 9 would decrease the activity of the cationic form HB^+ of a weak base and increase the activity of the anionic form A^- of a weak acid. Both extracellular activity changes at constant intracellular activity could hyperpolarize the peritubular cell membrane, if permeable to these ionic species. Alternatively, in pH 9 the extracellular concentration of uncharged weak base B and uncharged weak acid HA increases and decreases, respectively, with corresponding intracellular changes through nonionic diffusion, leading to an increase of intracellular pH. Two electrical consequences of this nonionic diffusion could be either a change in pH_i-dependent ionic conductances or a change in diffusion potential of the charged buffer species due to titration of the intracellular buffer.

Insofar as the electrical effects of changes in extracellular activity of charged buffer ion species are concerned, two parameters should be considered: first, the transference number for that ion, i.e. a combination of permeability coefficient and average absolute membrane concentration of the ion, and second, the magnitude of the change in concentration gradient of the buffer ion across the membrane achieved by the alteration in pH. In solutions a, b and c quantitatively the most important buffer

Table 5. Effect of changes of buffer concentration at constant pH 7.5

Perfusion solution a	3.2 mM Tris-H^+/0.8 mM Tris
V_1^c	−43.0 ± 1.3 (25)
V_3^c	−5.2 ± 1.3 (5)
Perfusion solution j	**32 mM Tris-H^+/8 mM Tris**
ΔV_1^p "on"	−1.5 ± 0.5 (2)
ΔV_1^p "off"	+1.0 ± 0 (2)
ΔV_3^p "on"	0
ΔV_3^p "off"	0
ΔV_1^l "on"	−0.8 ± 0.4 (8)
ΔV_1^l "off"	+1.8 ± 0.3 (4)[a]
ΔV_3^l "on"	0
ΔV_3^l "off"	0
ΔV_1^{pl} "on"	−1.1 ± 0.5 (17)
ΔV_1^{pl} "off"	+1.5 ± 0.4 (17)[a]
ΔV_3^{pl} "on"	−0.6 ± 0.6 (5)
ΔV_3^{pl} "off"	0

[a] Significant change in potential difference $P < 0.01$.

pair is Tris-Tris-H^+ (pK = 8.076). Other weak acids such as lactate and butyrate have a pK of 3.86 and 4.81, sufficiently below the range of the experimental pH values such that no appreciable changes in extracellular A^- are elicited.

Although it has been shown that Tris penetrates erythrocytes slowly [60], it is possible that its migration across the renal cell membrane may influence the change in membrane potential difference, seen during pH changes. Firstly, alkalinization to pH 9 decreases the cationic form of the buffer Tris-H^+ in the extracellular compartment about 8 times from 3.2 to 0.4 mM. Secondly, the concentration of the uncharged form of Tris increases from 0.8 to 3.6 mM during alkalinization, and penetration of this form into the cell would alkalinize the intracellular compartment. This rise in pH_i could interfere with membrane conductances. Thirdly, protonation of Tris entering the cell would to a variable degree increase intracellular concentration of Tris-H^+, depending on the extent of the final pH_i reached. For instance, if pH_i is 7.43 in nominally bicarbonate-free Ringer's at pH_o of 7.5 [10], intracellular Tris-H^+ would initially be 3.72 mM at nonionic diffusion equilibrium. At pH_o = 9.0, assuming ideal pH_i regulation at 7.43, nonionic diffusion of Tris would increase the intracellular Tris-H^+ concentration to 15.8 mM. The combination of extra- and intracellular concentration changes of Tris-H^+ would predict a maximum shift of Tris-H^+ diffusion potential from −4.1 mV in pH_o = 7.5 to −91 mV in pH_o = 9.0. More modest alterations of Tris-H^+ diffusion potential would occur if pH_i is allowed to alkalinize.

In order to simulate the effect of changes in Tris-H^+ diffusion potential, the effect of a sudden increase of buffer concentration at a constant pH of 7.5 was studied. A tenfold increase of the total Tris and Tris-H^+ concentration was achieved by substitution for sodium. The effects of acute one-sided and bilateral exposure of the tubule epithelium to high-buffer concentration are summarized in Table 5. Only the withdrawal of the high-buffer concentration causes small significant changes in the peritubular membrane potential difference when either the luminal or both sides of the tubular epithelium had been perfused with the test solution. These findings are in contrast with depolarizations of 10 mV reported when Tris substituted for 45 mM of Na [82]. The changes in potential difference of about 1.5 mV in our experiments are much smaller than and opposite in sign to those expected from a diffusion potential of the cation Tris-H^+. Therefore an altered diffusion potential for Tris-H^+ cannot have been the sole mechanism in eliciting the pH-dependent potential changes in Table 3. This conclusion is based on the assumption that the diffusion potential for Tris-H^+ in the experiment of Table 5 did not dissipate completely as otherwise would be the case in perfect pH_i regulation.

The experiments of Table 5 could also simulate intracellular alkalinization since any penetration of nonionic Tris and its protonation would exceed at least initially the influx of Tris-H^+. If the membrane potential responds also to intracellular alkalinization the minor effects of ΔV_1^l and ΔV_1^{pl} in Table 5 may be caused in fact by an internal pH effect rather than by the buffer ion concentration gradient. Internal alkalinization cannot be the sole mechanism of the pH-dependent potential changes of Table 3, since nonionic penetration of Tris for the experimental solution j should at least equal that for solution b, but solution b hyperpolarizes V_1 more extensively. However, it is not possible to exclude some role of the cation Tris-H^+ in these observations, if a primary hyperpolarization due to a rise in intracellular pH would be canceled by the depolarizing effect of elevated permeant extracellular Tris-H^+.

In conclusion, hyperpolarization in pH 9 may be caused by any combination of the three following mediating effects: elevation of external pH, elevation of internal pH, change in Tris-H^+ ion diffusion potential, but could not be explained by an elevation in internal pH only or by a Tris-H^+ ion diffusion potential only. Finally, it should be noted that changes in diffusion potential for the anions, lactate, butyrate, phosphate and perhaps bicarbonate cannot be excluded as the intracellular activity change cannot be properly estimated without quantitative knowledge of pH_i changes.

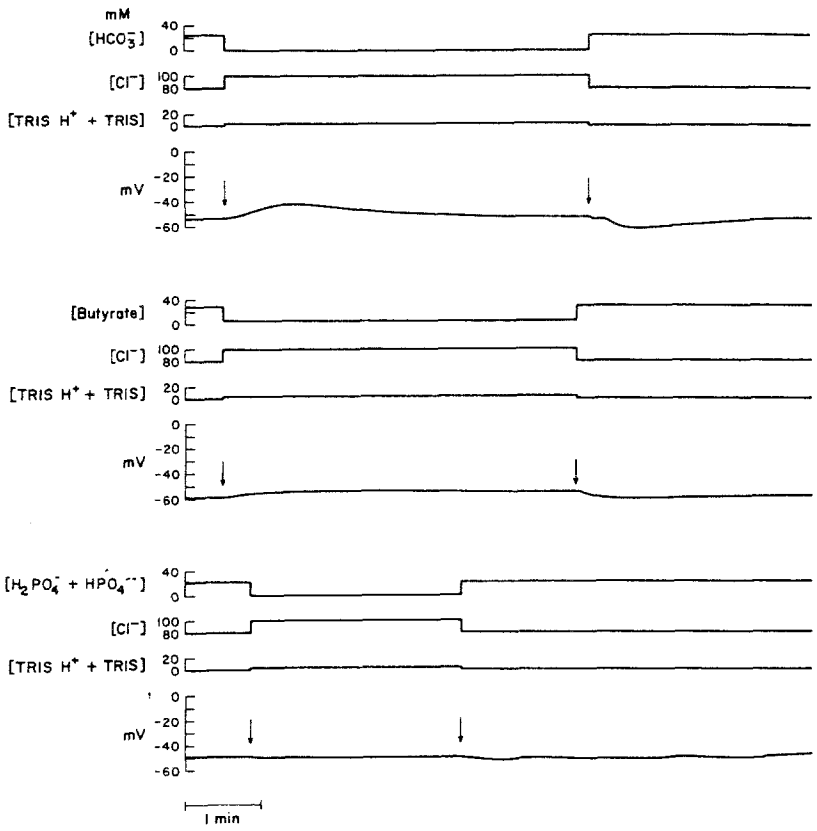

Fig. 4. Different panels showing the response of V_1 when bicarbonate (upper), butyrate (middle) and phosphate (lower) were suddenly removed from the peritubular perfusion solution and substituted by Cl^- at a constant pH of 7.5

The Role of Buffer Permeability in Determining Electrical Parameters

The preceding section did not establish the unique role of extracellular pH at the exclusion of intracellular pH or of buffer ion diffusion potential effects in the pH control of peritubular membrane potential. Since these two factors, which could not be ruled out, depend on the permeability of both charged and uncharged forms of buffers, it is important to evaluate the role of buffer permeability in determining electrical parameters of the proximal tubule cell.

Kidneys were pre-equilibrated with weak-acid buffer solutions, k, l and m at relatively high concentrations of 25, 28 and 22 mM of bicarbonate, butyrate and phosphate, respectively. Indeed, in order to test the role of intracellular pH it is essential to pre-equilibrate the tubule cell with a buffer species at high concentration in order to predict qualitatively changes in intracellular pH elicited by extracellular maneuvers (Fig. 4). In addition, the three buffers were chosen to be of different pK_a in order to generate a different intracellular concentration of the nonionic species (HA). Removal of the weak-acid buffer from the extracellular compartment thus leads to a variable diffusion gradient of the nonionic species depending on the pK_a and presumably a variable degree of intracellular alkalinization, if the permeability of each HA were similar.

While variable degrees of intracellular alkalinization can be obtained with the three buffers used, when the weak-acid buffer is removed from the outside medium, a definite transmembrane concentration gradient of the ionized species of the buffer will exist possibly leading to an anion diffusion potential. Clearly, the experimental protocol always includes the possibility of both intracellular pH changes and ion diffusion potential effects. However, the protocol chosen was such that intracellular pH change (alkalinization) should hyperpolarize and the concomitant buffer anion gradient should depolarize the cell membrane. The protocol used is illustrated in Fig. 4. A long pre-equilibration time at high-buffer concentration of about 25 mM was allowed to reach sufficient intracellular distribution. Then the buffers were suddenly replaced by chloride in the peritubular perfusion fluid, the pH being maintained constant by a Tris-butyrate buffer in low concentration, while the peritubular membrane potential difference was monitored. Figure 4 depicts the effect of a sudden replacement of these three buffers on V_1. As shown in the upper part of the Figure an instantaneous transient depolarization occurs immediately followed by a partial repolarization, when all HCO_3^- is replaced by Cl^-. Subsequent

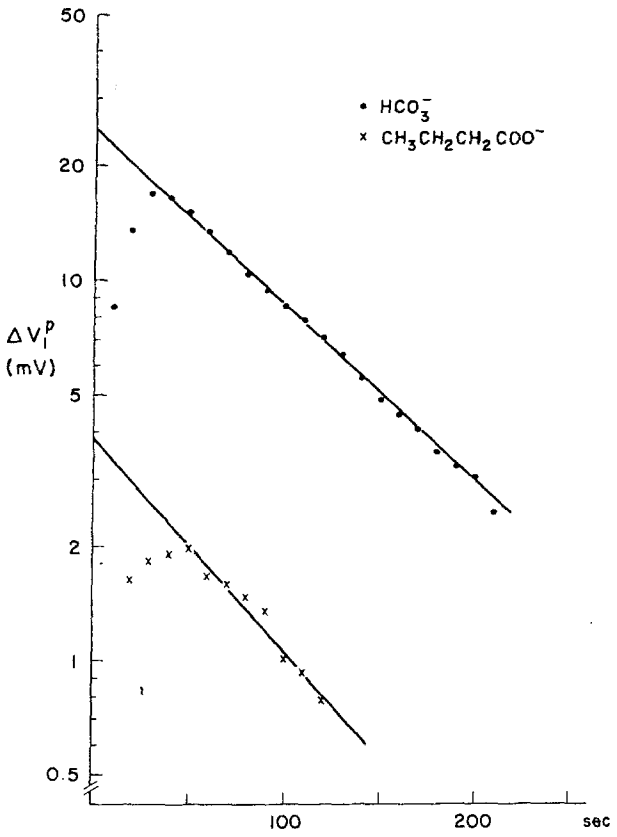

Fig. 5. The plot represents a maximal depolarization (ΔV_t^p) due to the sudden peritubular removal of bicarbonate and butyrate, respectively, at constant pH, when the potential displacement was graphically extrapolated to time zero

restoration of the original HCO_3^- concentration causes a mirror-image-like transient hyperpolarization from which the potential difference returns to the control level. When butyrate is suddenly decreased in the peritubular fluid, represented in the middle panel of Fig. 4, a much smaller transient depolarization is elicited and the repolarization is slower and less complete. Practically no changes are observed when phosphate is removed as shown on the bottom of the Figure.

Apparent transference numbers for the buffer anions can be calculated from these potential transients as shown in the Appendix. Since the intracellular buffer anion concentrations are likely to decrease following extracellular removal of the buffer the initial depolarization expected from HCO_3^- or butyrate removal was calculated from extrapolation of the potential transient to time = 0 as shown in Fig. 5. Apparent transference numbers for the buffer anions are given in Table 6, using the analysis described in the Appendix. The results of these substitution experiments indicate that $t_{HCO_3^-} > t_{butyrate^-} > t_{(H_2PO_4^- + HPO_4^{2-})}$. Clearly, the calculation of apparent transference numbers by means of Eq. (A.2) yields the fractional current carried by the transport of the charged buffer species. However, the transference number does not imply solely movement of the ionized buffer species through conductive channels but also describes rheogenic symport of buffer anions associated with net transfer of negative charge.

The transport numbers of Table 6 may be somewhat underestimated since in all cases at the same time as buffer anion concentration was decreased, chloride concentration was increased by 20 mM. For a value of $t_{Cl} = 0.2$ [12] this altered chloride gradient would have contributed a hyperpolarization of −1.13 mV. To test this possibility the peak depolarization was assessed during the same concentration gradient change for bicarbonate but at constant chloride concentration, when phosphate, the least permeable buffer, substituted for bicarbonate. In this condition the concentration gradient for both buffer anions is about the same. Therefore the potential displacement will mainly be determined by the difference in permeability for bicarbonate and phosphate. A representative experiment is depicted in Fig. 6. The average peak ΔV_t^p is +9.98 ± 0.75 mV ($n = 29$), which is significantly different from 7.9 mV when bicarbonate was replaced by chloride ($P < 0.05$). Assuming $t_{(H_2PO_4^- + HPO_4^{2-})} =$ zero, another minimal estimate of t_{HCO_3} would thus be 0.123 if a sudden change in HCO_3^- external concentration from 25 to 1 mM is postulated. The steady-state potential displacement in this condition is +5.06 ± 0.83 mV ($n = 26$), which is also significantly higher than the value obtained with the chloride replacement. Both increments in peak ΔV_t^p and ΔV_t^p at time ∞ can be explained by a higher permeability for chloride than for phosphate.

The calculation of buffer anion transference numbers as shown in the Appendix assumes that during the buffer replacements of Figs. 5 and 6 potential changes were not due to alterations in intracellular pH. As mentioned above, removal of the nonionic species HA from the external medium should lead to intracellular alkalinization providing HA exits the cell initially faster than the ionic species A^-. Recovery of pH_i would be expected to occur as a result of several mechanisms: efflux of A^- starting to exceed efflux of HA, pH_i regulation and finally depletion of intracellular HA and A^-. However, the transient rise in pH_i could have two effects: a direct influence on ion channel opening and indirectly by altering the intracellular buffer anion concentration of buffer systems other than that being substituted externally. A low pK for the other weak acids greatly blunts the rise in anion concentration due to an increase in pH_i and moreover a lower intracellular and extracellular concentration of buffers other than that being tested, should make

Table 6. Transference number for buffer anions

	HCO_3^-	Butyrate$^-$	$H_2PO_4^-$ + HPO_4^{2-}
ΔC (mM)	25	25	22
Peak ΔV_t^p (mV)	+7.9 ± 3.4 (28)	+3.9 ± 0.43 (19)	−0.18 ± 0.97 (11)
Rate constant of repolarization (sec^{-1})	0.019 ± 0.002 (19)	0.014 ± 0.002 (7)	0
ΔV_t^p at $t = \infty$ (mV)	+1.0 ± 0.32 (25)	+2.9 ± 0.65 (15)	+0.5 ± 0.53 (8)
Extrapolated ΔV_t^p at $t = 0$ (mV)	+31.2 + 3.2 (19)	+10.1 ± 2.9 (7)	+1.0 ± 0.5 (6)
t_i (minimum estimate)[a]	0.10	0.07	≈0
t_i (maximum estimate)[b]	0.38	0.18	≈0

[a] Calculated from peak ΔV_t^p.
[b] Calculated from extrapolated ΔV_t^p at $t = 0$.

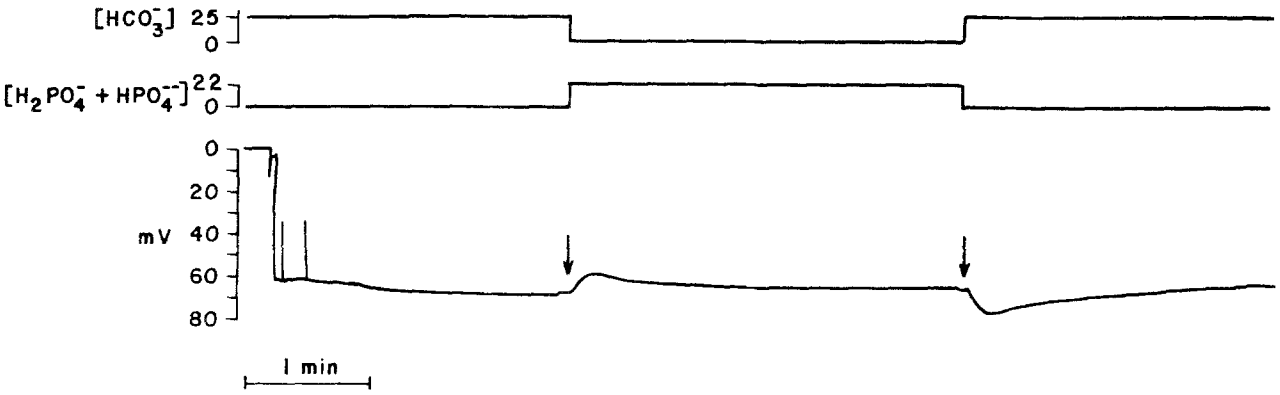

Fig. 6. Response of V_t, when bicarbonate was suddenly replaced by phosphate in the peritubular perfusion solution at a constant pH of 7.5

their effect on the membrane potential negligibly small. On the other hand, if intracellular pH influences membrane ion selectivity in a manner similar to extracellular pH, as hyperpolarization ΔV_t^p would be expected instead of the peak depolarizations observed. Therefore, although the magnitude of the contribution of intracellular pH by itself remains inconclusive, it is likely that the effective transference numbers for the buffer anions or their equivalent transported species in Table 6 are lower estimates.

Discussion

The present results confirm the observation by Anagnostopoulos [2] and Steels and Boulpaep [75] that a deviation from the normal pH in the perfusion fluid has a marked effect on the membrane potential difference in the *Necturus* proximal tubule. Yoshitomi and Hoshi [91] found a similar sensitivity of the membrane potential to intraluminal pH. These observations were extended in the present experiments by investigating the sidedness and the time course of the pH effect: a sudden increase or decrease of the pH on one or both sides of the cell at constant buffer concentration hyperpolarizes or depolarizes the tubule cell membrane, respectively. In the presence of a finite permeability to the ionic and nonionic components of the buffer, e.g. the hyperpolarization in pH 9 could result from i) an immediate external pH influence, ii) a buffer ion diffusion potential, itself changed immediately by the external buffer ion activity change and with some delay by the internal buffer ion activity change, or iii) a delayed component due to intracellular pH changes. However, the shift in potential difference occurs almost immediately considering the delay caused by unstirred layers of the interspaces at the basolateral membranes. The change in membrane potential ΔV_t^p as a result of peritubular pH changes was always monophasic as opposed to the triphasic response seen in isolated perfused *Ambystoma* proximal tubules [11], where pH changes were imposed at constant total HEPES buffer. The absence of a small overshoot of potential in the present ex-

periments may be due to a time course of adjustment of the intracellular buffer cation concentration which is fast compared to the unstirred layer delays in *Necturus* kidney. Alternatively, its absence may be due to a modest Tris-H^+ conductance. The slow potential change recorded in the third phase of the response to pH in *Ambystoma* proximal tubules may be absent in *Necturus* proximal tubules if no determinants of V_1 are critically dependent on pH_i. The change in membrane potential ΔV_1^l as a result of luminal pH changes was biphasic both in *Necturus* and in the report on *Ambystoma* [11].

In order to explore the role of buffer ion diffusion potentials and intracellular pH, in another set of experiments the effect of buffer concentration was determined at constant pH. In this case the extracellular component of the diffusion potential effect should precede the intracellular pH effect. Actual membrane potential changes were monophasic, fast and of small magnitude indicating that the contribution of intracellular pH and buffer Tris-H^+ diffusion potentials are negligible.

pH Affects Membrane Ion Channels and the Paracellular Pathway

At which cell border does external pH exert its effect, through which ionic mechanism, and what are the quantitative changes in membrane parameters at that cell membrane? In order to interpret the potential changes in terms of modifications of electromotive forces and membrane resistances at specific cell borders, potential differences were observed during three forms of identical pH alterations: unilaterally peritubular (p), unilaterally luminal (l) and finally, bilaterally (pl). Thus, for a given pH alteration, changes in peritubular and transepithelial potential difference were determined, i.e. ΔV_1^p, ΔV_1^l, ΔV_1^{pl}, ΔV_3^p, ΔV_3^l and ΔV_3^{pl}. These potential changes of Table 3 can then be interpreted according to the most simple model of the equivalent electrical circuit for the proximal tubule cell where no contribution of rheogenic pumping is assumed [16]. In this circuit the transepithelial potential difference V_3 is equal to the algebraic sum of peritubular and luminal potential difference:

$$V_3 = V_1 + V_2 \tag{2}$$

where subscript 1 refers to the peritubular membrane, 2 to the luminal membrane and 3 to the shunt pathway.

If the algebraic sum of the electromotive forces which exist at the three different ion pathways, i.e. E_1, E_2 and E_3, is not equal to zero a net current flow across each of the three barriers in the equivalent circuit arises. This closed-loop current I is given by the following equation:

$$I = \frac{E_1 + E_2 - E_3}{R_1 + R_2 + R_3} \tag{3}$$

where the R-terms represent the lumped ionic resistance at each barrier identified by the subscript. Since the loop current flows across all three barriers the potential difference across each barrier results from the sum of two terms: first the emf, E, and second the IR term. Thus,

$$V_1 = E_1 + IR_1 \tag{4}$$

$$V_2 = E_2 + IR_2 \tag{5}$$

$$V_3 = E_3 - IR_3. \tag{6}$$

It is clear from Eqs. (4) and (6) that observed ΔV_1 or ΔV_3 values may arise from parameter changes at the barrier facing the solution pH change, e.g. for ΔV_1 from a change in E_1 and R_1, but in addition from a change in the loop current I, which in turn depends on all six parameters E and R, for all three barriers in the circuit. Therefore the interpretation of observed ΔV_1 or ΔV_3 will be necessarily complex. Moreover each of the pH alterations p, l or pl is likely to affect the electrical elements of more than one barrier.

The purpose of recording potential difference changes during the identical pH changes on different sides of the epithelium is to use this information in order to systematically exclude the contributions of some parameter changes. The results of Table 3 indicate ΔV_3^l and ΔV_3^p to be of the same sign. This clearly shows that for the "l" change more is involved than an E_2 alteration and for the "p" change more than an E_1 alteration. If pH affects the permselectivity of a barrier it is reasonable to expect that a "p" pH change affects both E_1 and R_1, whereas an "l" change affects at least E_2 and R_2. Taking as an example the results of Table 3 for the "l" changes during alkalinization, $\Delta V_1^l = -5.45$ mV, $\Delta V_2^l = +4.69$ mV, $\Delta V_3^l = -0.76$ mV, a similar sign of ΔV_1^l and ΔV_3^l cannot be justified on the basis of a luminal membrane effect only, i.e. a change of E_2 and R_2. Indeed whatever the change in E_2 and R_2, this will only influence the values of V_1 or V_3 according to Eqs. (4) and (6) via a change in current I. For any given identical change in I, ΔV_1 and ΔV_3 should be of opposite sign. It may be concluded that other parameters outside E_2 and R_2 respond to luminal alkalinization. Barring changes in the parameters E_1 and R_1 of the contralateral cell membrane, assuming a delayed time course of intracellular activity changes, the most likely additional interaction of luminal pH could be on the paracellular shunt E_3 and R_3.

A positive ΔV_2^l suggests a positive ΔE_2^l. Hence ΔV_1^l can only be negative if ΔI is negative, i.e. the loop current decreases. A negative ΔV_3^l in the presence of a fall in I can only result from a rise in R_3 and/or a negative ΔE_3. Although it is not possible to quantitatively decide whether either R_3 or E_3 changes are involved, the following a priori reasons are considered. Two different mechanisms could alter E_3 during pH changes. This emf could behave as a hydrogen electrode. If the lumen is made alkaline or acid, ΔV_3^l should be positive or negative, respectively. In fact in Table 3 ΔV_3^l was negative for alkaline luminal fluid and positive for acid luminal fluid. Alternatively, E_3 probably originates from the salt concentration in the lateral intercellular space. Sackin and Boulpaep [66] have calculated a NaCl concentration ratio between interspace and lumen of 1.1 sufficient to explain the isotonic fluid reabsorption in the *Necturus* proximal tubule. If pH alters the paracellular ion selectivity, a negative ΔV_3^l at pH 9 would mean that t_{Cl}/t_{Na} is increased, whereas a positive value of ΔV_3^l at pH 6 would mean that t_{Cl}/t_{Na} is decreased. However, these postulated changes in t_{Cl}/t_{Na} are contrary to those expected from a titration of polar groups in the tight junction. Moreover, in previous experiments [76] a decrease of the t_{Cl}/t_{Na} ratio was observed at both pH 6 and 9. The sign of ΔV_3^l agrees with the change in t_{Cl}/t_{Na} observed earlier at pH 6, but not at pH 9. However, the absolute value of ΔV_3^l is not accounted quantitatively by ΔE_3^l if the NaCl ratio is only 1.1. Between pH 7.5 and 6 according to the observed change in t_{Cl}/t_{Na} [76], a maximum ΔE_3^l of +0.32 mV is predicted as opposed to the observed ΔV_3^l of +1 mV. Therefore it can be concluded that the observed ΔV_3 values are not a consequence of changes in E_3, which are probably negligible. In contrast, no theoretical limit can be placed on the magnitude of changes in R_3 with external pH. It is concluded that the observed signs of ΔV_1^l, ΔV_2^l, ΔV_3^l in pH 9 cannot be explained by a positive ΔE_2 and a ΔR_2 only. An auxiliary paracellular effect is required, most probably a rise in R_3.

Considerations similar to those applied above to the example of luminal pH changes, when applied to the effects of a change in peritubular pH, (Table 3), would lead to the conclusion that, most likely in addition to a change in E_1, either R_1 or R_3 is affected by peritubular pH. Finally, when pH is alkaline on both sides of the epithelium ΔV_1^{pl} is negative and of the same absolute magnitude as ΔV_2^{pl} which is positive. This finding is compatible with a change in electromotive forces of both membranes, which is equal and of opposite sign: $\Delta E_1^{pl} = -\Delta E_2^{pl}$. However, these results do not prove that the partial ionic conductances of both membranes are the same or equally sensitive to a given extracellular pH modification. It is important to emphasize that the need to invoke effects on additional electrical parameters rests on the assumption that intracellular activity changes did not contribute.

In view of the larger potential changes observed during peritubular pH changes, the ionic mechanism was investigated in more detail. In particular the apparent transference number for potassium at the peritubular cell membrane increases at pH 9 by a factor 1.16. It means that, if the intracellular concentration remains constant, the ratio of permeability coefficients at the two pH values $\frac{P_K\ (\text{pH } 9)}{P_K\ (\text{pH } 7.5)}$ is also equal to 1.16. Although rises of 8 to 12 meq in intracellular K activity have been reported in alkaline pH_o [47, 51], intracellular K activity was unaltered during the initial 2 min of external pH change in *Necturus* [51] and rabbit proximal tubule [7]. A dependence of K conductance or K permeability on extracellular pH was inferred from comparisons between measured membrane potential and electromotive force for potassium [7, 23, 63]. In *Necturus* proximal tubule the relationship between membrane potential and external HCO_3 or pH was found to depend on the steady level of extracellular potassium [51] and led to the indirect conclusion that the K transference number was reduced in acidosis. A contribution of a pH-dependent conductance to observed electrical potential changes was also not excluded in rat proximal tubule [18].

The question arises whether the K conductance change is sufficient to explain the observed ΔV_1^p at pH 9 assuming that P_{Na} and P_{Cl} do not change. The observed ΔV_1^p value is either for summer animals −10.78 mV, or for winter animals, −6.04 mV. The predicted effect of the alkalinization on the membrane potential can be calculated according to the Goldman equation and the change in apparent P_K of 1.16. Using values of intracellular concentrations, obtained by Spring and Giebisch [71] and permeability ratios measured by Boulpaep [13], a maximum hyperpolarization at pH 9 of −2.2 mV would be expected for winter animals and of −2.1 mV for summer animals.[1] These values are far below the observed magnitude of ΔV_1^p.

Firstly, one could argue that the calculation of

[1] Taking for winter animals cell concentrations of K 111.29, Na 28.81 and Cl 32.31 meq, and for summer animals K 81.85, Na 42.95 and Cl 50.34 meq, it is calculated that for $P_{Na}/P_K = 0.04$ and $P_{Cl}/P_K = 0.4$, using the constant field equation, V_1 in winter animals at pH 7.5 is −52.25 mV and at pH 9 is −54.47 mV, whereas in summer animals at pH 7.5, $V_1 = -38.89$ mV and at pH 9 = −41.01 mV. It should be noted that Tris-H^+ was omitted from the constant field equation. Indeed because of the low extracellular Tris-H^+ concentration the effect on V_1 should be minimal even if $P_{Tris\text{-}H^+}/P_{K^+}$ were equal to 1. Omission of H^+, OH^-, butyrate and other anions from the Goldman equation is likewise justified.

the potassium transference number yields a minimal value because the slope of the measured potential change due to the potassium increase in Fig. 3 is smaller than the real change in diffusion potential for potassium, since $\Delta E_1 = \Delta V_1 - \frac{R_1}{R_2}\Delta V_2$, assuming a constant ratio R_2/R_1 [16]. A depolarization of V_1 (more +) is always accompanied with a depolarization of V_2 (more −). Therefore the actual value of ΔE_1 to be used for calculation of the transference number exceeds the absolute value of ΔV_1. However, using ΔV_1 instead of ΔE_1, a similar error is made for the determination of the potassium transference number at pH 7.5 and 9, providing all resistances are not affected by the pH alteration [16]. Moreover, the t_K estimate could be higher when the K concentration changes are performed close to the control K concentration of 2.5 meq, if the relationship of instantaneous potential difference to potassium concentration is not linear between 2.5 and 20 meq. Again the same error is probably made at both pH values.

Secondly, the P_{Na}/P_K and P_{Cl}/P_K ratios could be altered by a change in pH. Thus a fall of P_{Na}/P_K to zero together with the observed rise in t_K would predict a ΔV_1^p of −7.5 mV. However, it is difficult to understand why a high pH would increase t_K but decrease t_{Na}. In addition, the results of Table 4 indicate that reduced Na concentrations, i.e. a low t_{Na}, do not affect the pH response. Postulating a drop of P_{Cl}/P_K to zero in pH 9 would predict a ΔV_1^p of −22.6 mV. This assumption is unlikely since experimentally a reduced Cl concentration in Table 4 does not influence the pH sensitivity of peritubular membrane potential.

Possible Role of Buffer Anions on Membrane Current

Whereas the results shown in Table 5 excluded Tris-H^+ diffusion potentials and elevation of internal pH as a major determinant of the hyperpolarization of the membrane potential in pH 9.0, uncertainties remain regarding the possible role of other weak-acid buffer systems. Despite external concentrations of buffer, anions A^- such as lactate, butyrate, phosphate and residual bicarbonate are expected to change minimally in high pH solutions, the concentration of the nonionic species HA, particularly for those buffers with a low pK, would drop by an order of magnitude in the alkaline solutions. The resultant efflux of intracellular uncharged species HA and a limited rise in pH_i due to pH_i regulation would predict intracellular A^- concentrations to fall in the experimental pH 9.0 condition below their value in pH 7.5. If a conductive pathway or a rheogenic symporter (carrying negative charge) exists for any of these charged buffer anions at any of the cell borders, hyperpolarization of the cell would result.

A rheogenic Na/HCO_3 symporter carrying Na^+, HCO_3^- and negative charge in the same direction was described in the basolateral membrane of *Ambystoma* proximal tubule by Boron and Boulpaep [11]. Similar mechanisms were subsequently reported for *Necturus* proximal tubule [39] and rat proximal tubule [1, 89]. A strong relationship between membrane potential and pH_i was reported in *Ambystoma* proximal tubule, where intracellular alkalinization resulted from depolarization [68]. This alkalinization induced by depolarization was found to derive from both a luminal Na-dependent lactate movement and a contraluminal Na/HCO_3 transport [69].

In the present experiments nominally HCO_3-free solutions would not allow rheogenic Na/HCO_3 transport to contribute considerably to the membrane current. In contrast, a role for rheogenic transport systems of lactate or butyrate cannot be ruled out on the basis of the present data. However, without monitoring pH_i, actual changes in transmembrane chemical gradient for these anions cannot be estimated.

The data of Table 3 show a higher degree of hyperpolarization for peritubular alkalinization than for luminal alkalinization. Although pH_i was not measured in the present investigation, some assymmetry in the behavior of the pH_i response was noted in *Ambystoma* proximal tubule (*see* Fig. 1*B* of reference 11) where a change in pH_o in nominally HCO_3-free HEPES solutions resulted in a larger change in pH_i, when the change in pH_o was applied to the bath than when applied to the lumen. A larger intracellular pH_i change in the peritubular maneuver could be caused by a more complete nonionic equilibration across the peritubular membrane. One would thus expect a larger ΔpH_i to result in a greater drop of A^- for a number of buffers in the cell. A larger transmembrane diffusion potential for A^-, e.g. for lactate, in the peritubular pH change than in the luminal pH change, would explain the larger ΔV_1^p than ΔV_1^l observed in Table 3.

Finally, the observed hyperpolarization could result from a pH-dependent activation of some rheogenic pump mechanism. Rheogenic sodium transport is an unlikely candidate since the change in potential is still present when the sodium concentration is very low. Moreover, because the (Na,K)-ATPase has a pH optimum at 7.4 [9, 26], the peritubular membrane potential difference in pH 9.0 should rather depolarize from reduced rheogenic

Na transport. For the hyperpolarization to result from rheogenic hydrogen ion pumping [8, 50], hydrogen translocation should be directed from cell interior to outside. However, increased proton extrusion is contrary to pH_i regulation in alkaline external solutions.

In conclusion, the observed pH-dependent changes in potential differences are partly explained by the effect of external pH on K channels. In addition, organic anions are likely to contribute to the membrane current.

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Appendix

Computation of Buffer Anion Transference Numbers

From the depolarizations and hyperpolarizations observed in Fig. 4, when weak-buffer solutions are removed from the extracellular compartments it is possible to calculate apparent transference numbers for the buffer anions. Since the peritubular capillaries were perfused at a physiological perfusion pressure the slow time course of the onset of the effect of buffer anion removal on the peritubular potential differences in Fig. 4 could be due to peritubular diffusion delays. Therefore the real peak depolarization could be larger than that observed if no redistribution had yet taken place. The maximal depolarization for HCO_3^- and butyrate removal that could have been observed at time zero was therefore obtained by graphical extrapolation of the potential displacement to time zero as is shown in Fig. 5 for a typical measurement. The exponential time course of the repolarization suggests that changes in intracellular anion concentrations occur driven by the difference between the equilibrium potential for the buffer anion and the membrane potential difference. Since the initial concentration gradient of the anion across the peritubular membrane is thought to be responsible for the peak depolarization, a decrease of this gradient will repolarize the membrane with an exponential-like time course. As shown in Table 6 the rate constant of this repolarization when HCO_3^- leaves the cell is 0.019 sec^{-1} whereas it is smaller but not significantly different during the fall in concentration of butyrate: 0.014 sec^{-1} ($0.2 < P < 0.1$). The observed peak depolarizations ΔV_t^p upon exposure of the kidney to bicarbonate-free and low-butyrate solutions, respectively, are determined when the rates of concentration changes of the respective anions in the extra- and intracellular fluid are equal. This average peak change is $+7.9 \pm 3.4$ mV ($n = 28$) for the bicarbonate-free perfusion, whereas a smaller peak change of $+3.9 \pm 0.43$ mV ($n = 19$) is observed when butyrate is replaced by chloride. The change in V_t is not significantly different from zero when phosphate is removed from the perfusion solution. Since the recovery of the potential difference after the peak depolarization follows a single exponential function, it can be assumed that during this time course the buffer ion does not accumulate in the interstitial fluid. Recovery of the potential difference is then only determined by the change in intracellular concentration:

$$\frac{dV_t}{dt} = k \frac{d \ln([A_i^-]/[A_o^-])}{dt}. \qquad (A.1)$$

Therefore the maximum depolarization for a maximum concentration gradient at time zero can be extrapolated from this exponential course of the membrane potential recovery. The calculated extrapolated values of ΔV_t^p at time zero are $+31.2 \pm 3.2$ mV ($n = 19$) and $+10.1 \pm 2.9$ mV ($n = 7$) due to the instantaneous substitution of bicarbonate and butyrate, respectively. Because the potential difference did not recover completely a steady-state potential displacement was observed after 2 to 3 min of redistribution of the respective buffer anions. This ΔV_t^p at time infinity was $+1.0 \pm 0.32$ mV ($n = 25$) for the bicarbonate replacement, which is significantly different from zero and $+2.9 \pm 0.65$ mV ($n = 15$) for the butyrate replacement.

From the instantaneous depolarizations it is possible to calculate transference numbers for the different anions according to the following equation:

$$\frac{\delta V}{25.25\delta \ln[A^-]_o} = t_{A^-} \tag{A.2}$$

where $[A^-]_o$ is the extracellular buffer anion concentration. A maximal estimate of this number in Table 6 assumes that the buffer anion is washed out of the extracellular compartment to a finite low and constant value at time zero and that the depolarization corresponds to the extrapolated value. A minimal estimate of transference number in Table 6 assumes that no intracellular buffer anion is washed out by the time of exponential recovery in Fig. 4 and therefore the same concentration gradient as above is used. Since bicarbonate was completely deleted from the perfusion solution the exact concentration in the immediate vicinity of the membrane at the moment of the replacement is unknown. In order to calculate t_{HCO_3} different gradients can be assumed. If after Cl-Tris butyrate substitution still 1 mM of HCO_3^- remains in the outside solutions t_{HCO_3} is estimated from the extrapolated peak ΔV_t^p to be 0.38. The minimum t_{HCO_3} estimate of 0.10 is based on the observed peak ΔV_t^p and a remaining extracellular concentration of 1 mM. At constant pH, transport numbers for HCO_3 were observed ranging from 0.41 to 0.63 in *Necturus* proximal tubule [55], 0.27 in frog proximal tubule [57] and 0.68 in rat proximal tubule [19]. A sizeable permeability for HCO_3 in *Necturus* proximal tubule was inferred from electrical changes induced by stilbenes [28] and also reported in rabbit proximal convoluted and straight tubule [6] as well as in rat proximal tubule [90]. For computing the transference number for butyrate the concentration step goes ideally from 28 to 3.2 mM. For this gradient and the extrapolated ΔV_t^p value a maximum estimate of $t_{butyrate}$ of 0.18 is calculated. The minimum estimate is 0.07. Since no change in V_t occurs with phosphate removal, the transference number is zero for either of the ionic phosphate species.