J. hysiol. (1977), **267**, *pp.* 571–599
With 7 text-figures
Printed in Great Britain

KINETICS OF LUMINAL ACIDIFICATION IN CORTICAL TUBULES OF THE RAT KIDNEY*†

By G. GIEBISCH‡, G. MALNIC†
G. B. DE MELLO† and M. DE MELLO AIRES†,

From the Departments of Physiology, Instituto de Ciencias Biomedicas, University of Sao Paulo, Sao Paulo, Brazil, and Yale University School of Medicine, New Haven, Connecticut, U.S.A.

(*Received 7 June 1976*)

SUMMARY

1. Some kinetic aspects of renal tubular acidification were studied in proximal and distal tubules of the rat kidney by combining stationary microperfusion methods and continuous measurements of luminal pH changes of phosphate or bicarbonate buffers by means of antimony electrodes. The analysis included the measurement of steady-state pH, steady-state buffer concentrations and acidification half-times. From these data, net rates of tubular bicarbonate reabsorption and of H ion secretion were obtained since it was shown that the rate of phosphate acidification provides a realistic estimate of H ion secretion.

2. Experiments were performed in control rats, in animals undergoing metabolic acidosis or alkalosis and in control and acidotic rats receiving the carbonic anydrase inhibitor Diamox.

3. In all experiments, the rates of tubular bicarbonate reabsorption and of phosphate acidification (H ion secretion) were proportional to luminal buffer levels. The change of luminal acid concentrations followed first-order kinetics.

4. Steady-state transepithelial pH differences were reduced in metabolic alkalosis and after diamox but augmented during metabolic acidosis.

5. Acidification half-times were prolonged in metabolic acidosis and after Diamox but remained similar to control levels in metabolic alkalosis.

6. From the observation that both bicarbonate reabsorption and

* Supported by grants from Fundacao de Amparo a Pesquisa do Est. Sao Paulo, Cons. Nac. Des. Cient. Tecnol. and the NIH, NSF and the American Heart Association.

† Department of Physiology, Instituto de Ciencias Biomedicas, Sao Paulo, Brazil.
‡ Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510, U.S.A.

phosphate acidification are similarly affected by these experimental manoeuvres, it is concluded that H ion secretion plays a key role in both transport processes.

INTRODUCTION

The ability of the mammalian kidney to acidify the urine maximally is limited to a pH of not lower than 4·0–4·5, and these transepithelial pH gradients are achieved only at the most distal nephron sites. Hence, only very small amounts of H ions exist in free form in the tubular fluid. Net acid excretion is thus dependent on the availability of buffers in the tubular fluid. To achieve maximal rates of urinary H ion excretion, phosphate salts are usually given and represent the most abundant buffer species in the final urine (Pitts & Alexander, 1945; Schiess, Ayer, Lotspeich & Pitts, 1948; Swan & Pitts, 1955).

The present study deals with a quantitative analysis of intratubular titratable acid formation and compares the latter with bicarbonate reabsorption. Stationary microperfusion methods were combined with continuous measurements of pH changes of luminally deposited buffers (Malnic & Mello Aires, 1971; Malnic, Mello Aires, de Mello & Giebisch, 1972*b*). This approach offers some advantages over free-flow measurements of acid-base parameters in buffer-loaded animals. In particular, variations in H ion secretion that could be due to alterations in the filtered buffer load and the associated extracellular volume expansion are avoided (Giebisch & Windhager, 1973; Kurtzman, 1970; Rector, 1973).

In this study, both the mechanism of acidification of phosphate and bicarbonate buffers, as well as the effects thereupon of carbonic anhydrase inhibition and of several acid-base disturbances, were explored. It was observed that under all acid-base disturbances, the rate at which the luminal steady-state pH was approached depended on the nature of the luminal buffer and luminal acid accumulation followed first-order kinetics. Thus, net acidification rates were uniformly proportional to luminal buffer levels. Both the rate of pH changes in the lumen, the steady-state luminal pH levels and the rates of buffer acidification were significantly affected by acid-base disturbances and by carbonic-anhydrase inhibition. These findings are discussed with respect to the participation and possible mechanisms of tubular H ion transport. Parts of this material have appeared in summary form in *Pflügers Arch. ges. Physiol.* **331**, 275–278, 1972, and in *Kidney int.* **1**, 280–296, 1972.

METHODS

Male albino rats ranging in weight from 250 to 350 g were anaesthetized by sodium pentobarbitone (40 mg/kg I.P.). Several groups of animals were used. (1) Control rats were maintained on a standard rat pellet diet. During the experiment, they received

an infusion of 3% mannitol in isotonic saline at a rate of 0·1 ml./min. (2) In a second group of rats, metabolic alkalosis was induced by the infusion of a 5% $NaHCO_3$ solution at a rate of 0·1 ml./min. At least 1 hr elapsed between the start of the infusion and the first microperfusion. (3) A third group of animals received the carbonic anhydrase inhibitor acetazolamide (Diamox, prime: 20 mg/kg, maintenance: 20 mg/kg.hr, given in isotonic $NaHCO_3$ at a rate of 0·1 ml./min). This rate of bicarbonate infusion has been found to maintain plasma bicarbonate at near normal levels. (4) A state of metabolic acidosis was achieved in another group of rats by keeping them for at least 3 days on a control diet to which 4% $CaCl_2$ was added. In addition, their water supply was substituted by a solution containing 70 mM-$CaCl_2$. Also, during the experiment, they received a mixture of NH_4Cl 150 m-equiv/l. in 3% mannitol at a rate of 0·1 ml./min. (5) In a fifth group of rats, the effect of acetazolamide during metabolic acidosis was studied. These animals received a priming dose of 20 mg/kg of this drug and a maintenance dose of 20 mg/kg.hr which was added to the NH_4Cl-mannitol mixture as used in the fourth group.

The preparation of the animal and the micropuncture techniques followed methods previously described (Malnic, Klose & Giebisch, 1964; Malnic & Mello Aires, 1970; Malnic, Mello Aires & Giebisch, 1972*a*). Blood and urine pH were measured anaerobically by means of a model EA 520 Metrohm capillary glass electrode. Total CO_2 in blood and urine were determined with a Natelson microgasometer.

Tubules were perfused by means of double-barrelled micropipettes, one barrel containing Sudan-black coloured castor oil, the other either a 100 mM-Na_2HPO_4 or a 100 mM-$NaHCO_3$ solution, both of which had an initial pH in excess of 8·0 and were made isosmotic by addition of raffinose. In animals treated with Diamox, tubular bicarbonate solutions were pre-equilibrated with 5% CO_2 in air, since under these circumstances CO_2 equilibration in split-drops had previously been found to be markedly delayed (Malnic & Mello Aires, 1971). The castor oil was used to isolate the perfusion fluids from the rest of the tubular contents by methods which also have been described in detail previously (Gertz, 1963; Malnic & Mello Aires, 1970, 1971). Early and late distal tubules were localized by the appearance time of lissamine green in the perfused segments (Wright, 1971). An antimony micro-electrode and a Ling–Gerard reference micro-electrode, or, alternatively, a double-barrelled Sb/reference electrode were introduced into the fluid column and the electrical potential difference between the two electrodes, which is proportional to luminal pH, was measured by a Keithley model 604 differential electrometer and recorded on a Beckman model RS Dynograph.

The preparation and use of the antimony micro-electrode has been described in detail in several papers from our laboratories (Malnic & Mello Aires, 1971; Malnic, Mello Aires & Cassola, 1974; Malnic *et al.* 1972*a*; Vieira & Malnic, 1968). Recently, some authors have observed lower pH values in bicarbonate/CO_2 solutions than theoretically expected or measured with glass electrodes, particularly when calibrations were done with non-bicarbonate (e.g. phosphate) buffers. For instance, Karlmark found that in such situations, pH values measured with the Sb electrode were 0·16 units lower than theoretical values (Karlmark & Sohtell, 1973). Somewhat larger deviations were found by Puschett & Zurbach (1974) and Green & Giebisch (1974). On the other hand, Kunau did not observe significant differences between measurements made with Sb and glass micro-electrodes when measuring the pH of bicarbonate-containing solutions (Kunau, 1972). In the present study, only a small pH difference was observed between measurements made with the antimony or a macro glass-electrode. In a series of 66 measurements of a 25 mM sodium bicarbonate solution equilibrated with 5% CO_2 in air, performed with forty-two micro-electrodes, 59% of the obtained pH values were within $\pm$0·1 pH unit of the glass electrode

values, and 85% within 0·2 pH units. The mean of all measurements was 0·066 ± 0·0178 pH units lower than the mean value obtained with the glass electrode. This difference is significantly different from zero ($P < 0·01$). In terms of bicarbonate concentrations, this error corresponds to a 15% difference in bicarbonate concentration. We chose not to correct for this small error. In our experiments 50 mM phosphate buffers, prepared according to Hawk, Oser & Summerson (1954) were used for pH calibrations. This is within the concentration range used in the present phosphate perfusion experiments. In these latter measurements, a bicarbonate error should be minimal. It is apparent that in the present series of experiments, bicarbonate has only a small effect on the accuracy of the Sb electrode.

From the time course of the pH change the concentration of NaH_2PO_4 was calculated by the Henderson–Hasselbalch equation using a pK of 6·8. For the case in which a constant concentration of luminal buffer was assumed, the NaH_2PO_4 concentration at time t is given by the expression (see Appendix I):

$$(NaH_2PO_4) = \frac{100}{10^{(pH-6·8)}+1}, \quad (1)$$

where 100 is the total buffer concentration and pH the measured value at time t. (NaH_2PO_4) increases with time and reaches a steady-state level $(NaH_2PO_4)_\infty$. To calculate acidification rates, the log of $(NaH_2PO_4)\infty - (NaH_2PO_4)_t$ was plotted against time in seconds. In contrast to pH changes, such a plot generated a straight line, indicating that the NaH_2PO_4 concentrations approach their steady-state value in an exponential manner. The half-time ($t_{\frac{1}{2}}$) of the approach of acid phosphate concentrations to their steady-state value was calculated from the slope of these lines. A similar approach, described in detail in a previous paper (Malnic & Mello Aires, 1971), was used to calculate the half-times of bicarbonate reabsorption. All calculations were made using a Fortran programme and an IBM 360 computer.

The present approach to measuring tubular acidification rates depends upon evaluation of the half-times or rate coefficients ($k = \ln 2/t_{\frac{1}{2}}$) of pH changes and the final steady-state acid phosphate or bicarbonate concentrations. During phosphate perfusions, acid phosphate concentrations (initially equal to zero due to injection of a solution containing only Na_2HPO_4) rise due to H ion secretion and reach stable steady-state levels. Assuming constant total phosphate concentration, the rate of the approach of acid phosphate concentration to this steady-state level will be proportional to the driving force, i.e. the difference between steady-state NaH_2PO_4 (As) and NaH_2PO_4 concentration at time t (A):

$$-d(As-A)/dt = k_1(As-A). \quad (2)$$

Maximal transfer rates of H ion secretion can be calculated from the rate coefficients of acidification, the respective driving force and tubular geometry according to

$$J_H = \frac{\ln 2 . r . As}{2 . t_{\frac{1}{2}}},$$

where J_H is the rate of H ion secretion (n-mole.cm^{-2}.sec^{-1}), and r is the luminal radius expressed in cm (Gertz, 1963; Malnic & Mello Aires, 1971; Malnic *et al.* 1972*b*). Fig. 1 shows a plot of $(NaH_2PO_4)_\infty - (NaH_2PO_4)_t$ as a function of time which, at $t = 0$, reduces to $(NaH_2PO_4)_\infty$.

In a separate series of stationary microperfusion experiments, relative volume changes of the luminal fluid samples and chloride concentration changes were measured in experiments in which a 100 mM-Na_2HPO_4 solution was used as perfusion fluid. The change of length of the fluid column was measured by means of an

ocular micrometer at 5–10 sec time intervals. The chloride concentration change was measured by recollection of the perfusate after varying time intervals and analysis of the collected perfusate for chloride by the method of Ramsay, Brown & Croghan (1955).

An approximation of the standard errors of functions of experimentally determined data was obtained by the method given by Eisenberg & Gage (1969).*

RESULTS

Table 1 summarizes acid-base data of the different groups of animals. Blood pH as well as plasma bicarbonate levels were significantly increased in metabolic alkalosis, and reduced in metabolic acidosis. Similarly, urine pH was increased in metabolic alkalosis as well as in animals receiving acetazolamide. In metabolic acidosis urine pH and bicarbonate levels were reduced. Rats in metabolic acidosis receiving acetazolamide Diamox had urinary pH and bicarbonate values not different from animals in control acid-base conditions. It should be noted that the alkalinizing effect of Diamox on urine pH is sharply reduced in metabolic acidosis.

Fig. 1 summarizes a representative set of data obtained during stationary perfusions with phosphate buffers in proximal and distal tubules in a control, in a 5 % sodium bicarbonate, and in a Diamox-treated rat. Two points deserve mention. Firstly, it is apparent that, generally, the change of luminal acid phosphate concentrations, when plotted semi-logarithmically as function of time, generates a straight line and thus follows first-order kinetics, in both proximal and distal tubules. Stated

* For instance, standard errors of H (J_H) and bicarbonate (J_{HCO_3}) transport rates were obtained from the variances of steady-state acid phosphate and bicarbonate concentrations and of half-times of acidification. The covariance term was disregarded, i.e. it was assumed that these parameters varied independently. According to Eisenberg & Gage (1969) the variance (s_z^2) of a function Z of two variables, x and y, with experimentally determined variances s_x^2 and s_y^2, is given by

$$s_y^2 = \left(\frac{\partial z}{\partial x}\right)^2 s_x^2 + 2\left(\frac{\partial z}{\partial x}\right)\left(\frac{\partial z}{\partial y}\right) \mathrm{cov}_{xy} + \left(\frac{\partial z}{\partial y}\right)^2 sy^2. \quad (3)$$

In the present series of experiments, we have disregarded the covariance term x and y ($\mathrm{cov}_{xy} = 0$). Using this relationship, the variance of H ion transport (J_H) as a function of H_∞ and $t_{\frac{1}{2}}$ is:

$$sy^2 = \frac{a^2(t_{\frac{1}{2}}^2 . s_H^2 + H_\infty^2 . s_{\frac{1}{2}}^2}{(t_{\frac{1}{2}}^2)^4}$$

where

$$a = \frac{\ln 2 . r}{2} \quad (r = \text{tubular radius})$$

The standard error of J is given by

$$Se_J = \left[s_J^2\left(\frac{1}{n_H} + \frac{1}{n_{t_{\frac{1}{2}}}}\right)\right].$$

TABLE 1. Acid-base parameters in different experimental groups

Group	Blood pH	Plasma HCO_3^- (m-equiv/l.)	Urine pH	Urine CO_2 (m-equiv/l.)
Control	7·38 ± 0·011 (33)	21·0 ± 0·47 (35)	6·11 ± 0·045 (27)	3·86 ± 0·32 (15)[a]
Metab. alkalosis	7·59 ± 0·018 (13)**	43·9 ± 3·25 (12)**	8·02 ± 0·045 (16)**	252·3 ± 15·3 (16)**[a]
Diamox	7·33 ± 0·015 (30)	22·9 ± 1·30 (21)	7·89 ± 0·068 (24)**	—
Metab. acidosis	7·01 ± 0·033 (26)**	7·2 ± 0·66 (23)**	5·94 ± 0·042 (21)**	1·75 ± 0·11 (29)**
MA + Diamox	6·87 ± 0·031 (17)**	6·6 ± 0·57 (16)**	6·11 ± 0·075 (26)	3·79 ± 0·80 (24)

Means ± S.E. (no. of obs.) ** ($P < 0·01$; [a] (from ref. 21),

differently, the rate of acid phosphate generation, a process which will be shown to be largely due to tubular H ion secretion, is proportional to the luminal acid phosphate concentration. Similar results have been obtained in stationary microperfusion experiments with respect to the kinetics of tubular bicarbonate reabsorption (Malnic & Mello Aires, 1971). Secondly, it can be seen from inspection of Fig. 1 that in proximal and distal tubules both the rate of approach to steady-state luminal phosphate levels as well as the absolute level of steady-state acid phosphate concentrations (the ordinate intercept), are subject to modifications by the acid-base status of the animals' body fluids.

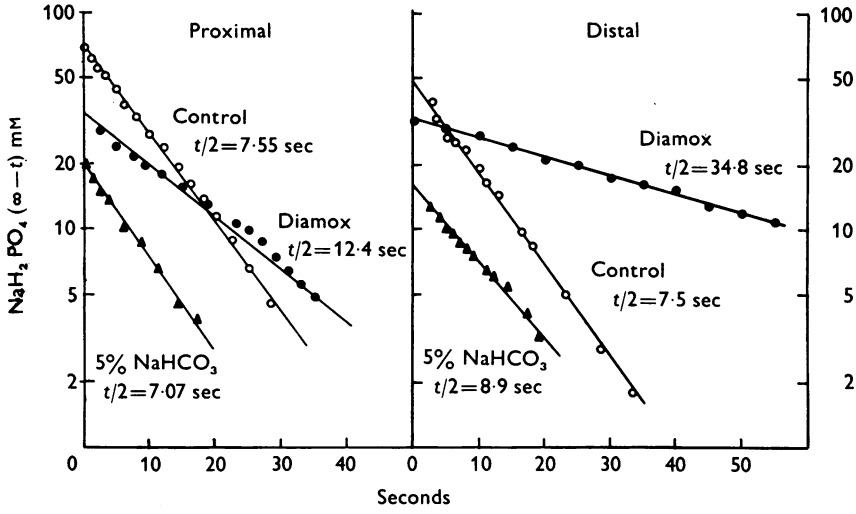

Fig. 1. Representative examples of proximal and distal tubular approach of acid phosphate to its steady-state level ($NaH_2PO_{4_\infty}$) in control animals and in animals receiving 5% $NaHCO_3$ or Diamox.

In Fig. 2, representative examples of results of proximal and distal tubular perfusions with phosphate in metabolic acidosis, with and without Diamox, are compared to results of similar perfusions in control animals.

The mean values of steady-state pH, steady-state acid phosphate concentrations and the rate of change of luminal pH, expressed as half-times, in the different experimental conditions are summarized in Table 2. Significant rates of tubular acidification takes place in control animals in which steady-state luminal pH values lower than arterial blood were observed. Half-times of acidification in both proximal and distal tubules were of the order of 7–8 sec. During induction of metabolic alkalosis and in Diamox-infused rats steady-state pH values in proximal tubules rose

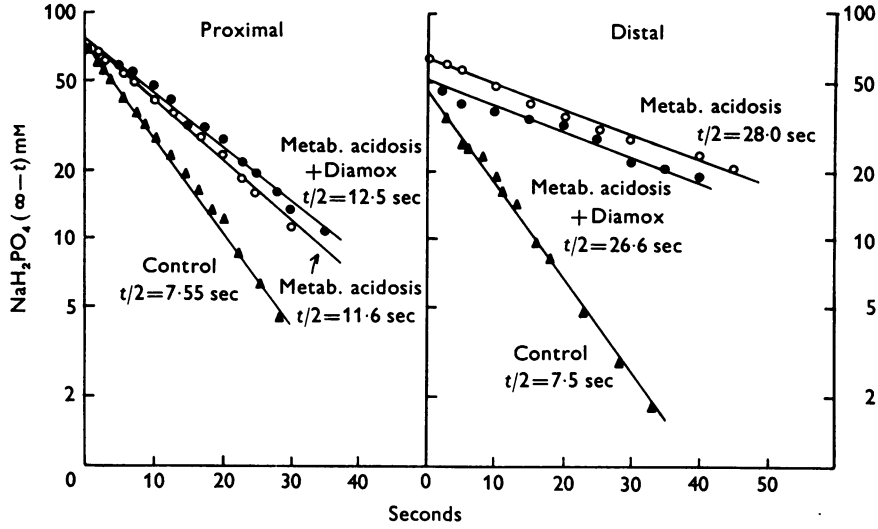

Fig. 2. Representative examples of proximal and distal tubular approaches of acid phosphate to its steady-state level ($NaH_2PO_{4\infty}$) in control animals and in animals during metabolic acidosis, with and without Diamox.

TABLE 2. Steady-state pH (pH_∞) and acid phosphate concentrations (H_∞), and acidification half-times, in different experimental conditions. Luminal perfusion with 100 mM phosphate

		Means ± S.E. (no. of obs.)		
	Loc.	pH_∞	H_∞ (m-equiv/l.)	$t_{\frac{1}{2}}$ (sec)
Control	P	6·23 ± 0·031 (40)	74·1 ± 1·14 (40)	7·43 ± 0·24 (40)
	ED	6·88 ± 0·046 (27)	43·4 ± 2·37 (27)	8·01 ± 0·54 (26)
	LD	6·38 ± 0·143 (10)	65·3 ± 3·09 (10)	8·56 ± 1·21 (10)
5% $NaHCO_3$	P	7·00 ± 0·020 (17)**	36·6 ± 1·01 (17)**	6·85 ± 0·46 (17)
	ED	7·43 ± 0·064 (15)**	16·3 ± 1·87 (12)**	8·45 ± 0·83 (14)
	LD	7·39 ± 0·075 (19)**	20·9 ± 2·49 (18)**	7·32 ± 0·62 (19)
Diamox	P	7·07 ± 0·023 (23)**	33·3 ± 1·15 (23)**	13·2 ± 1·00 (22)**
	ED	7·03 ± 0·024 (19)	35·4 ± 1·23 (19)*	28·8 ± 2·54 (22)**
	LD	7·03 ± 0·055 (13)**	35·7 ± 2·69 (13)**	18·4 ± 1·96 (13)**
Metab. acidosis	P	5·93 ± 0·031 (44)**	86·5 ± 0·73 (44)**	10·1 ± 1·00 (33)**
	ED	6·11 ± 0·033 (24)**	81·5 ± 1·07 (24)**	25·5 ± 1·66 (31)**
	LD	5·94 ± 0·049 (19)**	86·0 ± 1·36 (19)**	14·4 ± 1·01 (15)**
Metab. acidosis	P	6·29 ± 0·037 (20)	75·1 ± 1·48 (20)	13·6 ± 1·01 (22)**
+Diamox	ED	6·15 ± 0·062 (13)**	79·9 ± 2·16 (13)**	29·6 ± 5·46 (13)**
	LD	6·17 ± 0·060 (13)	79·2 ± 2·09 (13)**	15·3 ± 1·60 (13)*

P, proximal tubule; ED, early distal; LD, late distal.
* $0·05 > P > 0·01$; ** $P < 0·01$. Statistical comparisons refer to controls.

significantly. In early distal tubules of control rats steady-state pH values were significantly higher than corresponding values in proximal tubules. They are further increased in metabolic alkalosis. Steady-state pH values were similarly elevated above control values in Diamox-infused rats in proximal, early and late disal tubules. Since final steady-state NaH_2PO_4-concentrations depend upon steady-state pH values, NaH_2PO_4 levels were significantly reduced in both alkalotic and acetazolamide-infused rats. As pointed out earlier, under control conditions the acidification half-times in both proximal and distal tubules were of the order of 7–8 sec. These values were not changed significantly in alkalotic rats, but were significantly prolonged in Diamox-treated animals. The difference is most striking at the early distal tubular level.

Inspection of Table 2 further shows that in both proximal and distal tubules during metabolic acidosis, steady-state pH values are significantly lower than in control animals. Accordingly, acid phosphate concentrations are elevated. Compared to control values, acidification half-times are, however, significantly prolonged in acidosis, especially so in distal tubules. Similarly, acidification half-times are also markedly slowed in rats receiving Diamox during metabolic acidosis. The effect of metabolic acidosis and of Diamox treatment on these half-time values is, however, not additive. This is apparent from the similarity of acidification half-time values in Diamox-treated animals and similarly treated animals undergoing metabolic acidosis. In the latter group of animals, steady-state pH values were only moderately elevated compared to untreated animals in metabolic acidosis. Accordingly, steady-state acid phosphate levels are only moderately reduced.

Table 3 summarizes the results of perfusion experiments with 100 mM bicarbonate in rats treated with Diamox and/or undergoing metabolic acidosis. These studies extend previous experiments in which it was demonstrated that after an initial period of CO_2 equilibration, the decrease in luminal bicarbonate concentration follows first-order kinetics (Malnic & Mello Aires, 1971). Mean control steady-state pH values are quite similar to those found during phosphate perfusions. Also, the direction of the pH changes observed during metabolic acidosis, with or without Diamox, is similar to those found during luminal phosphate perfusions. It should be noted that during control conditions acidification half-times are generally shorter when bicarbonate rather than phosphate constitutes the luminal buffer. These differences are less marked in animals treated with Diamox and/or in a state of metabolic acidosis.

Net H ion secretion rates (J_H) and bicarbonate reabsorption rates (J_{HCO_3}), are given in Table 4. It is noteworthy that bicarbonate transport rates exceed those of phosphate acidification in all experimental situations.

However, the directional changes occurring with respect to J_H and J_{HCO_3} are similar in the different acid-base disturbances.

In two additional series of experiments we obtained quantitative information of changes in intraluminal volume and buffer concentrations during

TABLE 3. Tubular acidification parameters in control rats and in different experimental conditions. Luminal perfusion with 100 mM sodium bicarbonate

Group	Loc.	pH_∞	$HCO_{3\infty}$ (mM)	$t_{\frac{1}{2}}$ (sec)
Control	P	6·21 ± 0·037 (56)	1·73 ± 0·24 (36)	5·15 ± 0·17 (89)
	ED	6·95 ± 0·077 (18)	11·6 ± 1·75 (18)	3·78 ± 0·13 (17)
	LD	6·60 ± 0·040 (18)	4·44 ± 0·34 (18)	4·98 ± 0·42 (16)
Diamox	P	6·83 ± 0·017 (56)**	7·10 ± 0·37 (56)**	8·12 ± 0·31 (34)**
	ED	6·85 ± 0·067 (21)	10·8 ± 1·98 (21)	25·8 ± 2·16 (22)**
	LD	6·93 ± 0·061 (18)**	10·6 ± 1·12 (18)**	17·9 ± 1·85 (18)**
Metab. acidosis	P	5·92 ± 0·039 (23)**	0·54 ± 0·052 (23)**	9·48 ± 0·62 (33)**
	ED	6·15 ± 0·044 (23)**	0·90 ± 0·10 (23)**	18·9 ± 1·55 (39)**
	LD	6·20 ± 0·039 (15)**	1·22 ± 0·12 (15)**	12·8 ± 1·39 (26)**
Metab. acidosis + Diamox	P	6·42 ± 0·028 (41)**	2·10 ± 0·11 (41)	17·9 ± 1·09 (19)**
	ED	6·30 ± 0·035 (23)**	1·71 ± 0·15 (23)**	29·9 ± 4·14 (19)**
	LD	6·18 ± 0·063 (19)**	1·44 ± 0·18 (19)**	18·2 ± 1·65 (19)**

For explanation of symbols, see Table 2. Statistical comparisons refer to controls. $HCO_{3\infty}$, steady-state bicarbonate concentration.

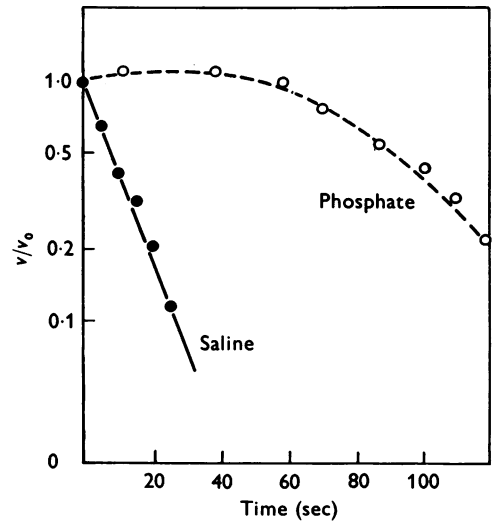

Fig. 3. Change of volume V (expressed as ratio of initial volume, V_0) of an initially chloride-free phosphate droplet in a control rat. Volume change of an isotonic NaCl-droplet is included for comparison.

phosphate perfusions. We assessed both volume and phosphate concentration changes in the following manner. Firstly, volume changes of phosphate solutions were measured in split-drop stationary perfusions identical to those used in the acidification experiments. Results from a representative phosphate perfusion experiment are shown in Fig. 3 and

TABLE 4. Net H ion secretion (J_{H+}) and bicarbonate reabsorption ($J_{HCO_3^-}$) calculated from acidification curves obtained during phosphate and bicarbonate perfusion respectively

Group	Loc.	J_{H-} (n-equiv/cm².sec)	$J^{-}_{HCO_3}$
Control	P	5·19 ± 0·26	9·92 ± 0·61
	ED	2·35 ± 0·29	10·13 ± 0·57
	LD	3·31 ± 0·69	8·31 ± 0·96
5% $NaHCO_3$	P	2·79 ± 0·28	—
	ED	0·84 ± 0·18	—
	LD	1·24 ± 0·26	—
Diamox	P	1·31 ± 0·15	5·95 ± 0·29
	ED	0·53 ± 0·07	1·50 ± 0·19
	LD	0·84 ± 0·15	2·16 ± 0·32
Metab. acid.	P	4·48 ± 0·59	5·45 ± 0·56
	ED	1·38 ± 0·13	2·27 ± 0·31
	LD	2·60 ± 0·25	3·34 ± 0·60
MA + Diamox	P	2·87 ± 0·32	2·84 ± 0·21
	ED	1·15 ± 0·31	1·42 ± 0·27
	LD	2·23 ± 0·34	2·35 ± 0·30

P, proximal tubule; ED, early distal; LD, late distal. Data calculated from mean values given in Tables 2 and 3, using eqn. (3). S.E. were calculated according to Eisenberg & Gage (1969) from the variances of the component means (H_∞, $HCO_{3\infty}^-$ and $t_{\frac{1}{2}}$); number of observations is given in Tables 2 and 3.

compared to a split-drop experiment in which isotonic saline was used. The rate of disappearance of saline follows simple exponential kinetics. In contrast, during perfusion with phosphate, the shape the curve relating relative volume changes to time indicates an initial period in which fluid enters the droplet. It is virtually certain that this volume increase is related to the initial transepithelial chloride gradient. Subsequently, the volume decreases due to egress of the limiting solute species, i.e. raffinose and phosphate. The mean half-time of reabsorption of this latter process was 51·8 ± 9·5 sec (n = 14). This value is quite similar to that previously found by Gertz (1963) during stationary perfusion experiments using raffinose solutions.

Since these volume changes may give rise to concentration changes of total phosphate in our stationary perfusion experiments, a second series

of experiments was carried out to measure the changes of intraluminal chloride concentrations in the initially chloride-free phosphate droplets. Under our experimental conditions it is safe to assume that chloride and phosphate make up most of the anionic content of the perfusate since the leakage of bicarbonate into the lumen contributes at most 3–5 m-equiv/l. (Bank & Aynedjian, 1967). In view of the isosmotic character of proximal tubular fluid, the rate of chloride entry into the lumen provides an estimate of tubular buffer (phosphate) dilution. It was observed that luminal chloride concentrations increase initially and reach a steady-

TABLE 5. Half-times of chloride inflow into initially chloride-free perfusion fluids

Solution	Proximal	Distal
$NaHCO_3$ control*	10·3 ± 1·69 sec (18)	—
Diamox*	15·5 ± 2·99 (17)	—
Na_2SO_4 control*	12·3 ± 2·34 (15)	—
Diamox*	14·8 ± 6·40 (15)	—
Na_2HPO_4 control	14·1 ± 2·00 (18)	20·1 ± 7·19 sec (12)
Diamox	—	26·5 ± 7·76 (10)

* Data from Malnic & Mello Aires (1970).

Half-times calculated from regression lines of ln $(Cl_\infty - Cl_t)$ against time, ± S.E. of mean of the slope (s_b).

state value after 70 sec. This approach of luminal chloride concentrations in phosphate droplets to the steady-state level was found to be exponential in nature. The half-time of chloride entry, obtained from a semilogarithmic plot of eighteen values of $Cl_\infty - Cl_t$ (where Cl_∞ and Cl_t are the chloride concentrations at steady-state levels and at time t, respectively), was 14·1 sec. Inspection of Table 5 indicates that this value is quite similar to the rate of chloride entry in proximal tubules during perfusion with other ions, including bicarbonate (Malnic & Mello Aires, 1970). Hence, these volume changes during which steady-state pH levels are reached are small since after 30 sec, the initial volume was reduced by only 11 %. Accordingly, such volume changes do not significantly affect the rate of tubular acidification as the half-time of this latter process is of the order of 7–8 sec (see Table 2).

DISCUSSION

The possible events which could lead to changes in H and phosphate ion concentrations in the tubular lumen are schematically depicted in Fig. 4. These are as follows. (1) Acidification by H ion secretion leads to titration of alkaline phosphate buffer to NaH_2PO_4. (2) NaCl and water

entry dilutes tubular contents and thereby lowers total phosphate concentration. The magnitude of this process is evaluated by the rate coefficient of chloride influx (see Table 5). However, the simultaneous dilution of *both* acid and alkaline phosphate salts does not change luminal pH. Hence, as shown in the Appendix this event does *not* introduce an error with respect to the calculation of acidification half-times. However,

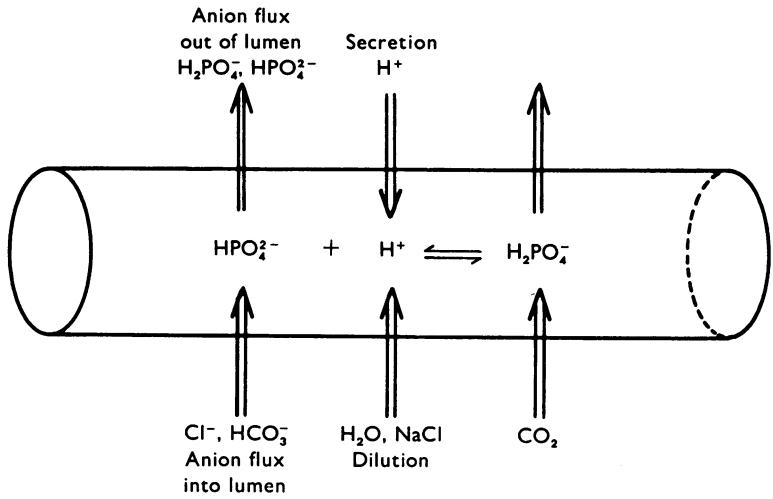

Fig. 4. Schematic representation of possible ion and fluid transfers during stationary perfusion with phosphate buffer which might lead to changes in luminal hydrogen and phosphate ion concentrations (see Discussion for detailed explanation).

inspection of eq. (3) indicates that such buffer dilution (reduction in As) will affect the calculation of the net rate of acidification: it will be reduced at times other than zero. For this reason, the acidification rates summarized in Table 4 are those obtained at t_0, i.e. calculated from the initial phosphate concentration and steady-state pH. (4) Phosphate loss out of the lumen by diffusion or active transport. Recently, Cassola (1974) has shown that the rate of ^{32}P loss from initially acid or alkaline solutions was 4–10 times slower than the rates of tubular acidification (3). Estimates based on a comparison of the respective rate coefficients of acidification and phosphate efflux indicate that loss of acid phosphate from the tubule may introduce an error of some 10 %. Hence, rates of tubular acidification may be underestimated by that amount if not evaluated at time t_0. The use of high luminal phosphate concentrations (100 mM) in the present series of experiments might also be questioned in view of its possible specific and potentially harmful effects on tubular transport processes. However, the fact that rate coefficients were found to be independent

of concentration constitutes evidence against such an interpretation. This view is supported by observations that the rate coefficients of acidification obtained when the lumen was initially perfused with 25 mM instead of 100 mM phosphate did not differ significantly from those of the present experiments ($t_{\frac{1}{2}}$ = 7·70 sec) (Mello Aires & Malnic, 1975). (5) Bicarbonate could diffuse into the phosphate droplets along its concentration gradient, and provide an additional proton acceptor that would not be measured by the acidification rate of phosphate. However, experiments in which peritubular capillary blood was replaced by bicarbonate-free phosphate Ringer have shown that this process is not an important determinant of luminal pH changes (Mello Aires & Malnic, 1975; Cassola, Giebisch & Malnic, 1977). Net acidification rates were not significantly altered by peritubular deletion of bicarbonate and CO_2. (6) Another aspect concerns the possibility that changes in luminal sodium concentration *per se* (depending on whether bicarbonate or Na_2HPO_3 was used) may change the rate of hydrogen ion movement. It has been reported that reduction of the luminal and peritubular sodium concentration may lower the rate of tubular acidification (Ullrich *et al.* 1975). However, significant reductions in acidification rates were found only below Na concentrations of 20 m-equiv/l., i.e. concentrations much lower than those used in the present experiments. In view of these considerations we believe that the rate of luminal phosphate acidification is a realistic estimate of tubular H ion secretion.

Two models of H ion secretion may account for the observed kinetics of tubular acidification (Malnic & Giebisch, 1972; Cassola *et al.* 1977). In the first, the H pump is thought to be independent of the H gradient against which it operates. In this model the rate coefficient of acidification represents the H ion conductance of the tubular epithelium.* First order

* Maximal rates of acidification (J_H) are obtained from eqn. (3) (Methods), which has the form

$$J_H = k.(Na_2HPO_{4_0} - Na_2HPO_{4_\infty}), \quad (4)$$

where k is the measured acidification rate coefficient and the second term the difference between initial and final buffer base concentrations. On the other hand, H ion permeabilities (P_H) could be obtained from the relation:

$$J_H = P_H.[H^+], \quad (5)$$

where J_H is the unidirectional H ion flow across the epithelium, and [H] the H concentration in the compartment originating this flow. From (4) and (5) we obtain:

$$k = \frac{P_H.[H^+]}{\Delta Na_2HPO_4} \quad (6)$$

indicating that k is a function not only of H ion permeability, but also of H ion concentration and buffer concentration difference. Hence, k has the meaning of a conductance rather than a permeability, since it is related to both permeability and concentration of the transferred ion species (see Cassola *et al.* (1977) for details).

kinetics reflect the fact that net H ion secretion is related to the difference between the activity of a constant secretory pump and a concentration-dependent H-leak. The latter reaches a maximum at steady-state pH levels when it equals the pump rate: net H secretion then is absent.

The second model, similar to that proposed by Steinmetz for the turtle bladder (Steinmetz & Lawson, 1971), assumes a negligible tubular hydrogen conductance and the presence of a gradient-dependent H pump. In this situation, the rate coefficient of acidification would provide an estimate of pump activity. At steady-state pH levels, active secretion would fall to zero; it would be maximal at the highest luminal buffer base load when opposing pH gradients are minimal.

It is likely that in a highly permeable structure as the proximal tubule, the first model might be a realistic one. In the distal tubule, an epithelial structure with higher resistance to ionic movement, the second model is thought to be more appropriate. However, our methods do not allow us to exclude rigorously the presence of both modes of operation at specific tubular sites. In the following our results will be discussed within the framework of the above described 'proximal' and 'distal' models of acidification. Some evidence bearing on this problem will be presented in another paper (Cassola *et al.* 1977).

Animals in metabolic alkalosis have similar rate coefficients (half-times) of acidification as animals in control acid-base conditions but significantly reduced steady-state acid phosphate concentrations (Table 2). The finding that the rate coefficient (acidification half-time) remains unaltered is consistent with the interpretation that the tubular leak (conductance) to H ions is unchanged in both proximal and distal tubules. The observed reduction in the net rate of acidification in metabolic alkalosis (Table 4) could, in a pump-leak system, be due to either an increased leak of H ions out of the lumen or to a decreased H ion pump activity. In the absence of a changed H ion leak the reduced rates of net acidification in the proximal tubule indicate reduced H ion pump activity. This could be due to diminished activity of the H ion pump or reduced availability of H ions. Since cell pH is likely to be elevated in alkalosis, we believe that reduced cellular H ion activity importantly participates in the fall of net acidification.

A similar interpretation applies to the distal tubular epithelium. Since net H ion secretion (J_H) depends on the rate coefficient (k) and the cellular H ion pool (H_c) ($J_H = X7H_c$) it is clear that in the presence of an unchanged rate coefficient of acidification, the cellular H ion activity is reduced in metabolic alkalosis. The present finding that at comparable luminal buffer loads, acidification in metabolic alkalosis is inhibited compared to control animals is not inconsistent with the well known load

dependence of proximal and distal tubular bicarbonate reabsorption under free-flow conditions (Malnic & Mello Aires, 1971; Malnic *et al.* 1972*a*; Malnic & Steinmetz, 1976; Kurtzman, 1970) particularly when care is taken to avoid extracellular volume expansion (Kurtzman, 1970; Rector, 1973). This is due to the fact that transport rates of bicarbonate ions depend both on the pump rate as well as on luminal substrate (buffer) concentration. If the latter is elevated more than the pump rate is reduced, over-all stimulation of net acidification will result.

The present study shows that Diamox, both at the proximal and distal tubular level, reduced the rate of H ion secretion (see Table 4). Acidification half-times were also significantly prolonged (Table 2). This observation suggests that H ion conductance is reduced by carbonic anhydrase inhibition. Since net acidification also falls, the pump activity must be reduced more than the H ion conductance since the latter change alone would not reduce net H ion secretion. Some findings support the notion that the cellular H ion pool is decreased after carbonic anhydrase inhibition. Firstly, Struyvenberg, Morrison & Relman (1968), using the DMO method, showed increased cell alkalinity after Diamox treatment. Secondly, Frömter (1975) and Garcia & Malnic (1976) have shown that the net transfer of base across the peritubular membrane is depressed after carbonic anhydrase inhibition. These observations suggest that the supply of H ions to a luminal pump mechanism is reduced after Diamox. With respect to the distal tubule, the observed reduction of net acidification can also be statisfactorily explained by reduced pumping of H ions since the rate coefficient of acidification is significantly depressed.

Earlier free-flow micropuncture studies as well as histochemical data have provided evidence that the carbonic anhydrase inhibitor Diamox acts at the proximal tubular level and reduces net bicarbonate reabsorption (Loennerholm, 1971; Vieira & Malnic, 1968). In contrast, the distal tubule may respond to the increased delivery of bicarbonate-containing solutions with enhanced bicarbonate reabsorption (Vieira & Malnic, 1968). It is most likely that the dramatically increased bicarbonate load is responsible for the enhancement of distal net reabsorption despite the fact that, as discussed above, the acidifying capacity of the distal tubular epithelium has been reduced by Diamox. A comparison between the free-flow distal buffer load and the epithelial capacity to reabsorb bicarbonate, as determined by the presently described methods, permits some insight into this matter. Normally, the distal bicarbonate concentration is of the order of 5 m-equiv/l. (Vieira & Malnic 1968). After Diamox it may increase sharply to levels some 15 times higher. Since (see Table 4) the rate of H ion secretion, at constant luminal buffer load, is decreased only by a factor of 5, it is clear that over-all net acidification may be enhanced

despite the inherent inhibition of distal tubular acidification. The fact that distal bicarbonate reabsorption, at constant luminal load, shows, after Diamox, a very similar proportional decline in comparison to H ion secretion into alkaline phosphate droplets, suggests that both processes are mediated by H ion secretion.

The data obtained during metabolic acidosis indicate the ability of both proximal and distal tubular epithelium to generate steeper maximal H ion concentration differences, albeit at prolonged acidification half-times. Inspection of Table 4 indicates that net acidification rates during phosphate and bicarbonate perfusions are moderately reduced. The $t_{\frac{1}{2}}$ data (Tables 2 and 3) strongly support the notion that acidosis induces some reduction in H ion conductance. However, in contrast to the situation in Diamox-infused rats, H ion pump activity must be reduced proportionately less than the leak of H ions out of the lumen since the steady-state concentration of acid phosphate is higher, and that of bicarbonate lower, than in control conditions (Tables 2 and 3).

These findings are of interest since they throw some light on the mechanisms by which the renal tubular epithelium responds to acidosis. Apparently, an important factor is a decreased leak conductance to H ions. This change would lead to increased efficiency of the tubular operation of acidifying the tubular fluid and the urine since even at lowered levels of H ion secretion rates the tubular mechanism of acidification is now rendered more effective in maintaining steeper transepithelial hydrogen ion gradients and thus also in titrating a relatively larger amount of tubular buffers. In the present series of experiments the H ion concentration differences across the proximal tubule wall, for instance, were almost doubled from a mean value of 620 n-equiv/l. in control rats to 1180 n-equiv/l. in acidotic rats. The moderately reduced net H ion secretory rate is still compatible with the lower pH values along the nephron in acidotic rats (Malnic & Giebisch, 1972; Gottschalk, Lassiter & Mylle, 1960) since the filtered load of bicarbonate can be markedly reduced during metabolic acidosis. Our findings are in agreement with those of Levine & Nash (1973) who observed a significantly decreased rate of bicarbonate reabsorption in acidotic rats when the filtered bicarbonate load was acutely increased.

On the other hand, considering the model for the distal tubule with relatively low passive permeability to H ions, the observed reduction in the rate coefficient of acidification would imply that the H ion secretory mechanism operates at a reduced rate but ceases to pump H ions at higher luminal H ion activities. The mechanism underlying this alteration in the kinetic parameters of distal tubular acidification during metabolic acidosis is presently unknown.

Maren and others have drawn attention to the fact that the diuretic

effect of acetazolamide is markedly reduced in acidotic animals (Brodsky & Satran, 1959; Maren, 1956, 1967, 1974). They have pointed out that during acidosis Diamox still leads to a highly significant reduction in the urinary excretion of titratable acid whereas it has little further effect on the rate of tubular bicarbonate reabsorption (Maren, 1956, 1967, 1974). This observation was interpreted to imply continuous reabsorption of bicarbonate by an acetazolamide-insensitive pathway which was not shared by the renal tubular mechanism responsible for the generation of titratable acid. Reabsorption of bicarbonate in its ionic form could be one of these mechanisms. We believe that another mechanism could also account for the differential effects of acetazolamide upon bicarbonate reabsorption and phosphate titration during acidosis.

Inspection of our data summarized in Table 4 indicates that *both* bicarbonate reabsorption as well as phosphate acidification are significantly depressed at the proximal tubular level by acetazolamide during metabolic acidosis. At the distal tubular level, the Diamox effects during metabolic acidosis are small as compared with those in otherwise normal animals (Table 4). Only at the late distal tubular level were steady-state pH and acid phosphate levels significantly ($P < 0{\cdot}01$) modified during phosphate perfusions (Table 2). During bicarbonate perfusions, Diamox exerted a significant effect ($P < 0{\cdot}01$) in increasing steady-state pH and bicarbonate levels only in the late distal tubule (Table 3). The proximal tubular inhibition of acidification after Diamox supports our view and that of others (Rector, 1973) that both bicarbonate reabsorption *and* phosphate acidification are due mainly to tubular secretion of H ions and thus are both susceptible to inhibition by Diamox. We believe that the finding of Maren (Brodsky & Satran, 1959; Maren, 1956, 1967), i.e. that during Diamox treatment bicarbonate reabsorption is less affected then titratable acid, i.e. phosphate acidification, could be due at least in part to the different properties of the bicarbonate and phosphate buffer systems. During bicarbonate perfusion titration is performed at constant acid (CO_2) concentration as steady-state conditions are approached. In contrast, phosphate titration takes place at nearly constant total buffer content. Thus, at comparable tubular pH levels the bicarbonate concentration has dropped to much lower levels whereas a relatively much larger fraction of the phosphate buffer remains as the alkaline salt (see Fig. 5).

Assuming a constant CO_2 level of 1 mM, a sample of perfusate originally containing 100 mM sodium bicarbonate would be reduced to 1·6 mM at a final pH of 6·3, according to the Henderson–Hasselbalch equation:

$$6{\cdot}3 = 6{\cdot}1 + \log \frac{B\ (\text{base})}{A\ (\text{acid})} .$$

At the same pH, a sample of 100 mM alkaline phosphate would be reduced to 24 mM

$$6{\cdot}3 = 6{\cdot}8 + \log \frac{(100-A)}{A}, \quad \text{where} \quad 100-A = B.$$

Thus, reaching this same steady-state pH, 98·4 % of the luminal bicarbonate would be reabsorbed, against the acidification of only 75 % of alkaline phosphate. The driving force for H ion secretion, $B_0 - B_\infty$ (see eqn. (3)) would be 98·4 mM for bicarbonate buffer, and 76 mM for phosphate buffer. This situation is shown in Fig. 5. Bicarbonate is titrated by the

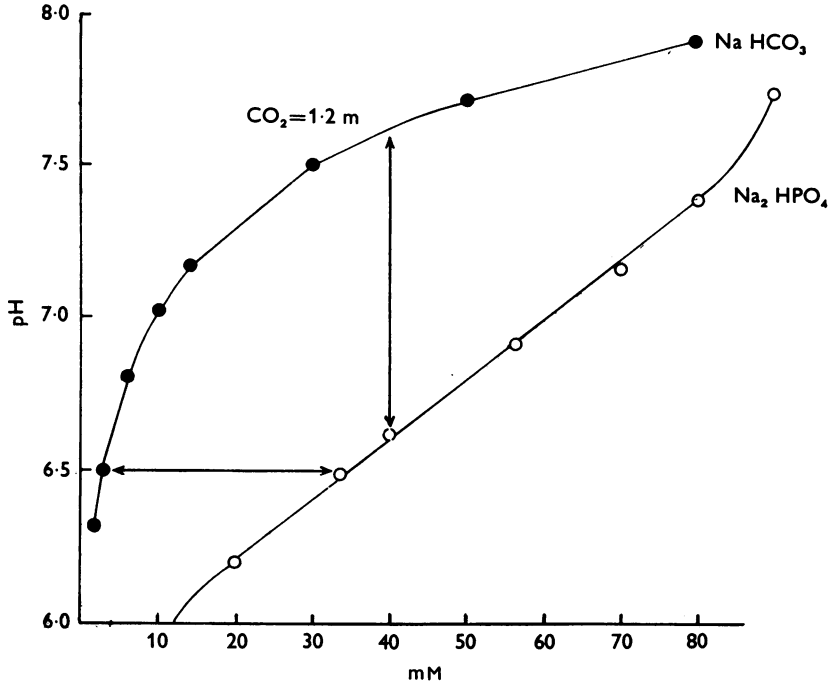

Fig. 5. Theoretical titration curves of bicarbonate and phosphate. pH changes were calculated from the Henderson–Hasselbalch equation for addition of acid at constant pCO_2 for bicarbonate, and at constant total buffer concentration in the case of phosphate. Initial buffer concentration: 100 mM. The plot of pH *vs.* concentration of the remaining buffer shows that for the two buffer species, at similar pH, widely different buffer base levels are found (horizontal arrow). Also, at the same buffer base concentration, pH differs markedly (vertical arrow).

tubules at constant acid (CO_2) concentration since the latter is normall fixed at about 1 mM. Accordingly, at urinary pH levels found in Diamox-infused rats (pH = 6·11, a value very close to the pK′ of the bicarbonate-CO_2 buffer system), bicarbonate has dropped to very low levels (see Fig. 5).

In contrast, phosphate buffer is titrated at constant buffer content and the phosphate buffer titration curve is considerably less steep in the pH range below 7·0 (see Fig. 5). Accordingly, urinary pH changes in the range found in acidotic animals with or without Diamox are much more effective in producing significant changes in urinary titratable acid, of which phosphate salts constitute the largest fraction, than they are in producing changes in bicarbonate concentration.

In addition, the observation that administration of Diamox during metabolic acidosis does not lead to a dramatic increase in urinary pH and bicarbonate excretion despite its significant inhibition of proximal bicarbonate reabsorption deserves comment. This finding must be due to the ability of nephron segments distal to the proximal convoluted tubule to reabsorb a large fraction of that moiety of bicarbonate which escaped proximal reabsorption in Diamox-treated acidotic animals. The finding that Diamox depresses distal tubular acidification less than in control animals, with respect to bicarbonate *as well as* phosphate buffers, is of interest. Although the underlying mechanism is not known, it may somehow be related to a larger carbonic-anhydrase independent H ion supply in acidotic animals. Since both bicarbonate reabsorption *and* phosphate acidification are reduced to a similar extent, particularly in the proximal tubule, our findings do not support a differential effect of this carbonic anhydrase inhibitor on bicarbonate reabsorption and titratable acid formation.

An additional important finding concerns the observation that acidification rates during phosphate perfusions, both in control and Diamox-treated animals, were consistently lower than those found during bicarbonate perfusion (see Table 4). This could be related to several factors. One is the possibility, during bicarbonate perfusions, of recycling of carbonic acid between lumen and cell (Rector, 1973). This mechanism is based on the assumption that non-ionized H_2CO_3 diffuses out of the lumen into the cell, maintaining an intracellular supply of hydrogen ions which is independent of carbonic-anhydrase stimulated CO_2 hydration. This mechanism would be operative only in the presence of bicarbonate, but not of phosphate since the latter is only poorly reabsorbed. A second element for the lower net acidification rates during phosphate as opposed to bicarbonate perfusions, in both the control and carbonic anhydrase inhibited state, could be the different behaviour of the two buffers during titration, already referred to above. A third factor which could contribute to the faster apparent acidification of luminal bicarbonate, compared to that of phosphate buffers, would be dilution of luminal bicarbonate by sodium and chloride (see Fig. 4). The driving force for this ion flow is the transepithelial chloride concentration gradient which develops as the lumen

is perfused with the initially chloride-free solution. The magnitude of this dilutional flow can be evaluated by the rate of chloride appearance during perfusion with bicarbonate containing solutions (Malnic & Mello Aires, 1970). The rate coefficients of the approach of chloride concentrations to their steady-state levels are compared to those of apparent acidification of bicarbonate and phosphate droplets in Table 6. It can be seen that the magnitude of the difference between the phosphate and bicarbonate rate coefficients is of the order of the chloride influx coefficient. This would provide a quantitatively satisfactory explanation for a large fraction of the observed difference between bicarbonate and phosphate acidification rates.

TABLE 6. Rate coefficients for acidification during perfusion with phosphate and bicarbonate buffers, compared to rates of chloride inflow, in proximal and distal tubules of rat kidney (sec^{-1})

	Perfusion		
Group	PO_4	HCO_3	Chloride inflow
Control, prox.	0·093 ± 0·0030 (40)	0·135 ± 0·0044 (89)	0·056 ± 0·0073 (18)
dist.	0·084 ± 0·0086 (36)	0·161 ± 0·0090 (33)	0·035 ± 0·019 (12)
Diamox, prox.	0·053 ± 0·0040 (23)	0·086 ± 0·0033 (34)	0·045 ± 0·0086 (17)

Rate coefficients of acidification calculated from $t_{\frac{1}{2}}$ values of Tables 2 and 3. Chloride inflow rate coefficients are means for a given segment and experimental condition calculated from data listed in Table 5.

It should be noted that the acidification rate of phosphate buffers in Diamox-treated rats is significantly higher than that calculated on the basis of uncatalysed CO_2 hydration. According to Maren (1967), the amount of Diamox administered in the present experiments is expected to block completely renal carbonic anyhdrase activity. Assuming a negligible back reaction ($H_2CO_3 \leftarrow H_2O + CO_2$), a CO_2 hydration rate coefficient (uncatalysed) of 0·15 sec^{-1} (Garg & Maren, 1972) and a mean blood CO_2 concentration of Diamox-infused rats of 1·35 mM (Table 1), the rate of uncatalysed CO_2 hydration is:

$$V_{CO_2} = k_{CO_2}.(CO_2) = 0{\cdot}15 \times 1{\cdot}35 = 0{\cdot}20 \text{ m-mole/sec.l.}$$

Since the cellular volume corresponds to about $\times 1{\cdot}5$ the luminal volume for a segment of proximal tubule, the rate of H ion generation by uncatalysed CO_2 hydration in tubular cells available for secretion into the tubular lumen is of the order of 0·30 m-mole/sec.l. This value is considerably smaller than the value observed in Diamox-treated animals of 1·75 m-mole/l. sec (see Table 6 in Cassola *et al.* 1976).

Several factors should be considered to account for the continued secretion of H ions after carbonic anhydrase inhibition during phosphate perfusions. Recently, Maren (1974) has proposed that the reaction

$$CO_2 + OH^- \rightleftharpoons HCO_3^-$$

is important at a pH above 8 and might contribute to neutralize OH^- formed by splitting water at the site of active H ion translocation. Conceivably, the OH concentration could reach levels high enough in a small restricted area to account for the observed uncatalysed rate of acidification (Maren, 1974). However, since the effective cellular OH^- concentration is unknown, it is difficult to assess the validity of this hypothesis.

Two additional sources of H ions should be considered after carbonic anhydrase inhibition. If active H ion extrusion continues across the luminal cell membrane, at a new steady-state, the cell interior should approach a lower electrochemical potential for H ions and could, accordingly, act as a sink for H ions. Thus, not only the luminal fluid (H_2CO_3 recirculation) but also extracellular fluid could contribute H ions to the cell pool. Since this fluid is in equilibrium with blood, it represents an additional source of H ion generation. Its actual effective magnitude, however, is difficult to evaluate. The recently demonstrated great sensitivity of luminal acidification rates to *peritubular* pH changes lends, however, support to this interpretation (Malnic & Giebisch, 1972; Mello Aires & Malnic, 1975). Finally, another factor contributing to the relatively high uncatalysed acidification rates during phosphate perfusions could be related to abnormally elevated cellular P_{CO_2} levels in Diamox-treated animals. Some evidence based on the finding of delayed CO_2 diffusion across the tubular epithelium in acetazolamide-treated rats (Malnic & Giebisch, 1972; Malnic & Mello Aires, 1971) is consistent with the view that, by an unknown mechanism, carbonic anhydrase accelerates the diffusional transfer of CO_2 across the cell wall of tubule cells. In the absence of an intact carbonic anhydrase system an increased level of cellular P_{CO_2}, due to diffusion delay of CO_2 out of the cellular compartment, could stimulate H ion secretion by generation of carbonic acid.

APPENDIX

1. Calculation of acid–phosphate levels

The Henderson–Hasselbalch equation,

$$\mathrm{pH} = \mathrm{pK} + \log B/A \tag{1a}$$

relates the concentrations of conjugate base and non-dissociated acid of a buffer system to its pK and pH. In terms of the total buffer concentration,

$$T = B + A \tag{1b}$$

we have

$$\mathrm{pH} = \mathrm{pK} + \log \frac{T-A}{A} \tag{1c}$$

Upon rearranging,

$$10^{\mathrm{pH}-\mathrm{pK}} = \frac{T-A}{A} \tag{1d}$$

and

$$A = \frac{T}{10^{\mathrm{pH}-\mathrm{pK}}+1}. \tag{1e}$$

Assuming that total phosphate concentration remains constant at a level of 100 mM and substituting a value of 6·8 for pK we obtain equation (1) in the body of the paper:

$$(\mathrm{NaH_2PO_4}) = \frac{100}{10^{\mathrm{pH}-6\cdot8}+1}.$$

2. The influence of total phosphate loss on acidification kinetics

It can be shown that

$$10^{\mathrm{pH}-\mathrm{pK}} = \frac{10^{\mathrm{pH}}}{10^{\mathrm{pK}}} = \frac{K_a}{(H)}, \tag{2a}$$

where K_a is the dissociation constant of the acid. From eqn. (1*e*) of Appendix 1, we obtain

$$A = \frac{T}{(K_a/H)} = \frac{T.H}{K_a+H}. \tag{2b}$$

At steady-state, the acid phosphate concentration is denoted as As and H as H_∞. Thus,

$$As = \frac{T_\infty}{(K_a/H_\infty)+1}. \tag{2c}$$

Dividing eq. (2*b*) by (2*c*) we obtain

$$As = \frac{\frac{T}{(K_a/H)+1}}{\frac{T_\infty}{(K_a/H_\infty)+1}} = \frac{(K_a/H_\infty)+1}{(K_a/H)+1} \cdot \frac{T}{T_\infty}. \tag{2d}$$

Rearranging,

$$As - A = As\left(1 - \frac{(K_a/H_\infty)+1}{(K_a/H)+1} \cdot \frac{T}{T_\infty}\right). \tag{2e}$$

Substituting

$$1 - \frac{(K_a/H_\infty)+1}{(K_a/H)+1} \cdot \frac{T}{T_\infty} = 1 - \frac{H(K_a/H_\infty)}{H_\infty(K_a+H)} \cdot \frac{T}{T_\infty} = y,$$

$$As - A = As.y, \tag{2f}$$

where y is a function of time. The rate of the approach of NaH_2PO_4 concentrations to its steady-state level is given by

$$\frac{d(As-A)}{dt} = -k(As-A) \tag{2g}$$

or, according to eqn. (2*f*),

$$\frac{d(As.y)}{dt} = -kAs.y. \tag{2h}$$

Assumption 1. If the total phosphate content of the tubule remains constant, As will also be constant; therefore, using a new rate constant, k_1, for this case, we have:

$$dy/dt = -k_1 y. \tag{2i}$$

Upon integration

$$y = y_0 e^{-k_1 t}. \tag{2j}$$

Under the present assumption and according to eqn. (2*e*), at $t = 0$, $y = y_0 = 1$. According to (2*f*), we obtain

$$y = \frac{As-A}{As} = e^{-k't} \quad \text{or} \quad A = As(1-e^{-k_1 t}). \tag{2k}$$

Assumption 2. If total phosphate content is not constant, the steady-state acid concentration will depend on time, since a steady-state pH may be reached before a steady-state phosphate concentration. Thus, we have:

$$\frac{dAs}{dt} = -k'[As - As_\infty] \tag{2l}$$

yielding

$$As = [As_0 - As_\infty]\, e^{-k\,t} + As_\infty.$$

Total phosphate loss, on the other hand, can be expressed as follows:

$$P = (P_0 - P_\infty)\, e^{-k't} + P_\infty \tag{2m}$$

where P is the phosphate concentration at time t, P_0 and P_∞ are phosphate concentrations at $t = 0$ (known) and at steady-state respectively (taken from Strickler, Thompson, Klose & Giebisch, 1964), and k' is the rate coefficient for total phosphate loss calculated from chloride influx. Using a mean half-time of chloride gain of 14·1 sec which corresponds to a rate coefficient of 0·049 sec^{-1}, the experimental data can be recalculated in terms of a progressively reduced total phosphate concentration of the perfusate. Using the Henderson–Hasselbalch equation

$$pH = pK + \log R,$$

where R is the salt/acid ratio:

$$R = 10^{(pH-pK)} \qquad (2n)$$

from which the acid concentration (A) can be obtained by the expression

$$A = P/(R+1),$$

where P is the corrected instantaneous phosphate concentration. The steady-state acid concentration (As) which is the asymptote towards which A tends, is not constant and will be given at time t by

$$As = P/(R_\infty + 1) \qquad (2o)$$

where $R_\infty = 10^{(pH_\infty - pK)}$.

Fig. 6 summarizes the time course of $P(Na_2HPO_4 + NaH_2PO_4)$ and A from a control experiment done on a proximal tubular segment. It is apparent that P declines exponentially during a time period in which A rises and approaches a steady-state level. A corrected rate coefficient for acidification (k_2) can be obtained from plotting log $(As - A)$ against time in sec. In this case, eqn. ($2h$) may be written

$$\frac{d(Asy)}{dt} = -k_2(As\,y). \qquad (2p)$$

Differentiating eqn. ($2p$) and evaluating at $t = 0$,

$$As_0\frac{dy}{dt_0} + y_0\frac{dAs}{dt_0} = -k_2(As_0 \cdot y_0). \qquad (2q)$$

At $t = 0$, total phosphate is constant and eqn. ($2i$) can be applied

$$\frac{dy}{dt} = -k_1 y \quad \text{or} \quad \frac{dy}{dt_0} = -k_1 y_0. \qquad (2r)$$

At $t = 0$, eqn. (21) becomes

$$\frac{dAs}{dt_0} = -k'[As_0 - As_\infty]. \qquad (2s)$$

Substituting eqns. (2r) and (2s) into (2q)

$$As_0(-k_1y_0)-y_0k'[As_0-As_\infty] = -k_2As_0y_0. \qquad (2t)$$

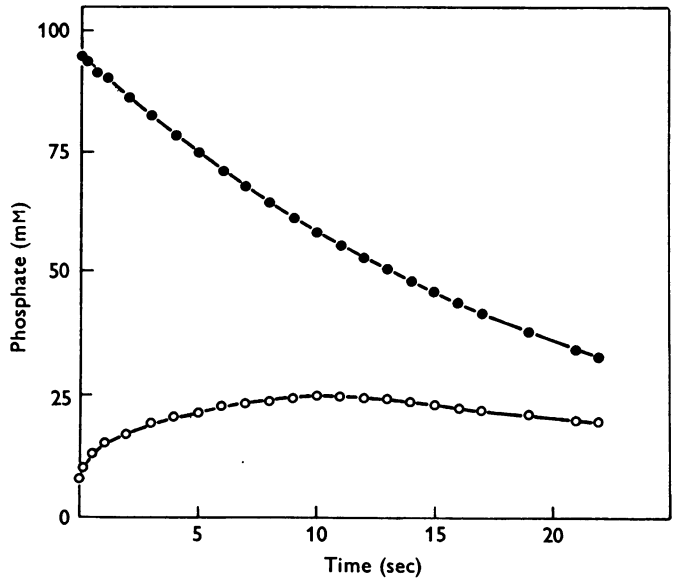

Fig. 6. Calculated change of total phosphate (●—●) and acid phosphate (○—○) concentrations with time in a proximal perfusion experiment.

Dividing by $(-y_0As_0)$ and rearranging

$$k_1 = k_2-k'[1-(As_\infty/As_0)]. \qquad (2u)$$

For $As_\infty \ll As_0$ we have:

$$k_1 = k_2-k'. \qquad (2v)$$

Fig. 7 depicts schematically the time course of the expected variations in total phosphate, acid phosphate concentrations and steady-state acid phosphate levels. In the upper part of the graph, a constant total concentration of phosphate (T) has been assumed, whereas in the lower part, both the total phosphate concentration and the steady-state acid phosphate concentration decay exponentially at a similar rate. Eqn. 2 (Methods) describes the time course of acidification for the case of a *constant* total phosphate concentration. The lower part of Fig. 7 shows the process of acidification during the fall of luminal phosphate concentrations. Here, eqn. (2q) describes phosphate loss during the perfusion period, in which As_0 and As_∞ are the level of the steady-state acid concentration at $t = 0$ and at final phosphate steady-state levels, and y is a function of k_1 of eqn. (2) and time.

It is clear from these considerations that the value of $As-A$ decreases

with time due to two phenomena: (1) a decrease due to phosphate egress and phosphate dilution, and (2) an increase in acid phosphate due to H ion secretion. Thus, the rate coefficient obtained from calculations based on both pH-changes and phosphate loss (k_2, see eqn. ($2p$)) is the sum of two coefficients, that of phosphate loss (k') and another (k_1) corresponding to acidification.

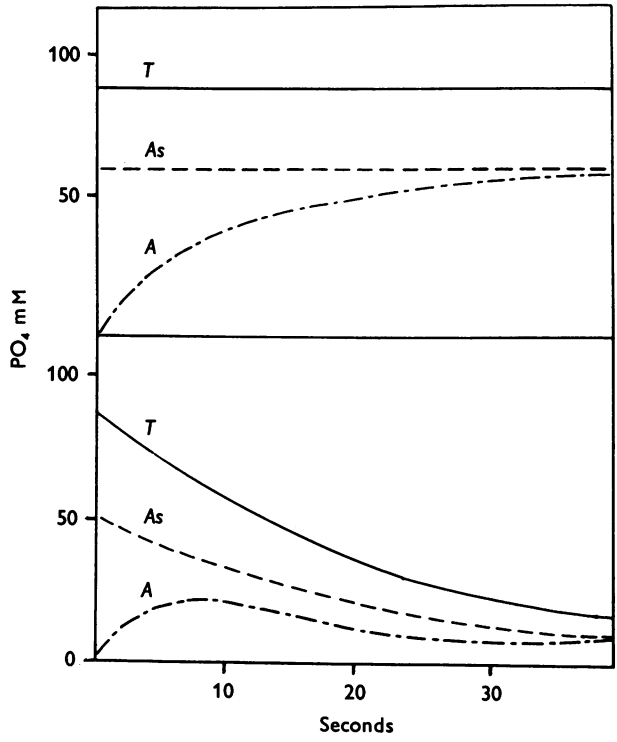

Fig. 7. Schematic representation of results of tubular phosphate acidification. In the upper panel, total phosphate concentration (T) has been assumed to remain constant (eqn. 2, Methods). In the lower panel, an exponential decay of the tubular total phosphate concentration has been assumed (eqn. 2, Appendix I). As, steady-state acid phosphate concentration; A, instantaneous acid phosphate concentration.

This relationship given in equation $2v$ indicates that the rate coefficient of H ion secretion can be estimated directly from k_1 since As_∞ is, in the present series of experiments, very far removed from As_0. The fact that eqn. ($2v$) may be used without introducing a significant error was confirmed by sample calculations ($n = 26$) in which ($k_2 - k'$) was not significantly different from k_1. Therefore, we have chosen to use half-times or rate

coefficients obtained by plotting eqn. (2), since this approach is independent of additional measurements and assumptions other than the determination of luminal pH as a function of time.

REFERENCES

BANK, N. & AYNEDJIAN, H. S. (1967). A microperfusion study of bicarbonate accumulation in the proximal tubule of the rat kidney. *J. clin. Invest.* **46**, 95–102.

BRODSKY, W. A. & SATRAN, R. (1959). Comparison of effects of acidosis and alkalosis on the renal action of Diamox. *Am. J. Physiol.* **197**, 585–594.

CASSOLA, A. C. (1974). Avaliacao da permeabilidade do epitelio de nefrons corticais a ions hidrogenio e fosfato. MS Thesis, Sao Paulo.

CASSOLA, A. C., GIEBISCH, G. & MALNIC, G. (1977). Mechanisms and components of renal tubular acidification. *J. Physiol.* **67**, 601–624.

EISENBERG, R. S. & GAGE, P. W. (1969). Ionic conductances of the surface and transverse tubular membranes of frog sartorius fibers. *J. gen. Physiol.* **53**, 279–297.

FRÖMTER, E. (1975). *Electrophysiological Studies on the Mechanism of H^+/HCO_3^- Transport in Rat Kidney Proximal Tubule.* Abstr. Symposia VI Int. Congr. Nephrol., Firenze, pp. 25–26.

GARCIA, E. M. & MALNIC, G. (1976). pH in cortical peritubular capillaries of rat kidney. *Pflügers Arch. ges. Physiol.* (in the Press).

GARG, L. C. & MAREN, T. H. (1972). The rates of hydration of carbon dioxide and dehydration of carbonic acid at 37° C. *Biochim. biophys. Acta* **261**, 70–76.

GERTZ, K. H. (1963). Transtubularere Natriumchloridfluesse und Permeabilitaet fuer Nichtelektrolyte im proximalen und distalen Konvolut der Rattenniere. *Pflügers Arch. ges. Physiol.* **276**, 336–356.

GIEBISCH, G. & WINDHAGER, E. E. (1973). Electrolyte transport across renal tubular membranes. In *Handbook of Physiology*, sect. 8, Renal Physiology, ed. ORLOFF, J. & BERLINER, R. W., pp. 315–376. Washington: American Physiological Society.

GOTTSCHALK, C. W., LASSITER, W. E. & MYLLE, M. (1960). Localization of urine acidification in the mammalian kidney. *Am. J. Physiol.* **198**, 581–585.

GREEN, R. & GIEBISCH, G. (1974). Some problems with the antimony microelectrode. In *Ion Selective Microelectrodes*, ed. BERMAN, H. J. & HEBERT, N. C., pp. 43–53. New York: Plenum.

HAWK, P. B., OSER, B. L. & SUMMERSON, W. H. (1954). *Practical Physiological Chemistry*, pp. 35–37. New York: Blakiston.

KARLMARK, B. & SOHTELL, M. (1973). The determination of bicarbonate in nanoliter samples. *Analyt. Biochem.* **53**, 1–11.

KUNAU, R. T. (1972). The influence of the carbonic anhydrase inhibitor Benzolamide (Cl-11,366) in the reabsorption of chloride, sodium and bicarbonate in the proximal tubule of the rat. *J. clin. Invest.* **51**, 294–306.

KURTZMAN, N. A. (1970). Regulation of renal bicarbonate reabsorption by extracellular volume. *J. clin. Invest.* **49**, 586–595.

LEVINE, D. Z. & NASH, L. A. (1973). Effect of chronic NH_4Cl acidosis on proximal tubular H_2O and HCO_3 reabsorption. *Am. J. Physiol.* **255**, 380–384.

LOENNERHOLM, G. (1971). Histochemical demonstration of carbonic anhydrase activity in the rat kidney. *Acta physiool. scand.* **81**, 433–439.

MALNIC, G. & GIEBISCH, G. (1972). Mechanism of renal hydrogen ion secretion. *Kidney Int.* **1**, 280–296.

MALNIC, G., KLOSE, R. M. & GIEBISCH, G. (1964). Micropuncture study of renal potassium excretion in the rat. *Am. J. Physiol.* **206**, 674–686.

MALNIC, G. & MELLO AIRES, M. (1970). Microperfusion study of anion transfer in proximal tubules of rat kidney. *Am. J. Physiol.* **218**, 27–32.

MALNIC, G. & MELLO AIRES, M. (1971). Kinetic study of bicarbonate reabsorption in proximal tubule of the rat. *Am. J. Physiol.* **220**, 1759–1767.

MALNIC, G., MELLO AIRES, M. & CASSOLA, A. C. (1974). Kinetic analysis of renal tubular acidification by antimony microelectrodes. In *Ion-Selective Microelectrodes*, ed. HEBERT, N. C. & BERMAN, H. J., pp. 84–108. New York: Plenum.

MALNIC, G., MELLO AIRES, M. & GIEBISCH, G. (1972*a*). Micropuncture study of renal tubular hydrogen ion transport in the rat. *Am. J. Physiol.* **222**, 147–158.

MALNIC, G., MELLO AIRES, M., DE MELLO, G. B. & GIEBISCH, G. (1972*b*). Acidification of phosphate buffer in cortical tubules of rat kidney. *Pflügers Arch.* **331**, 275–278.

MALNIC, G. & STEINMETZ, P. R. (1976). Transport processes in urinary acidification. *Kidney int*, **9**, 172–188.

MAREN, T. H. (1956). Carbonic anhydrase inhibition. IV. The effects of metabolic acidosis on the response to Diamox. *Johns Hopkins Hosp. Bull.* **98**, 159–183.

MAREN, T. H. (1967). Carbonic anhydrase: chemistry, physiology and inhibition. *Physiol. Rev.* **47**, 595–781.

MAREN, T. H. (1974). Chemistry of the renal reabsorption of bicarbonate. *Can. J. Physiol. Pharmac.* **52**, 1041–1050.

MELLO AIRES, M. & MALNIC, G. (1975). Peritubular pH and pCO_2 in renal tubular acidification. *Am. J. Physiol.* **228**, 1766–1774.

PITTS, R. F. & ALEXANDER, R. S. (1945). The nature of the renal tubular mechanism for acidifying the urine. *Am. J. Physiol.* **144**, 239–254.

PUSCHETT, J. B. & ZURBACH, P. E. (1974). Re-evaluation of microelectrode methodology for the in vitro determination of pH and bicarbonate concentration. *Kidney int.* **6**, 81–91.

RAMSAY, J. A., BROWN, R. H. J. & CROGHAN, P. C. (1955). Electrometric titration of chloride in small volumes. *J. exp. Biol.* **32**, 822–829.

RECTOR, F. C. (1973). Acidification of the urine. In *Handbook of Physiology*, sect. 8, Renal Physiology, ed. ORLOFF, J. & BERLINER, R. W., p. 431. New York: American Physiological Society.

SCHIESS, W. A., AYER, J. L., LOTSPEICH, W. D. & PITTS, F. R. (1948). The renal regulation of acid-base balance in man. II. Factors affecting the excretion of titratable acid by the normal human subject. *J. clin. Invest.* **27**, 57–000.

STEINMETZ, P. R. & LAWSON, L. R. (1971). Effect of luminal pH on ion permeability and flows of Na^+ and H^+ in turtle bladder. *Am. J. Physiol.* **220**, 1573–1580.

STRICKLER, J. C., THOMPSON, D. D., KLOSE, R. M. & GIEBISCH, G. (1964). Micropuncture study of inorganic phosphate excretion in the rat. *J. clin. Invest.* **43**, 1596–1607.

STRUYVENBERG, A., MORRISON, R. B. & RELMAN, A. S. (1968). Acid-base behavior of separated canine renal tubule cells. *Am. J. Physiol.* **214**, 1155–1162.

SWAN, R. C. & PITTS, R. F. (1955). Neutralization of infused acid by nephrectomized dogs. *J. clin. Invest.* **34**, 205–211.

ULLRICH, K. J., RUMRICH, G. & BAUMANN, K. (1975). Renal proximal tubular buffer-(glycodiazine) transport. Inhomogeniety of local transport rate, dependence on sodium, effect of inhibitors and chronic adaptation. *Pflügers Arch. ges. Physiol.* **357**, 149–163.

VIEIRA, F. L. & MALNIC, G. (1968). Hydrogen ion secretion by rat renal cortical tubules as studied by an antimony microelectrode. *Am. J. Physiol.* **214**, 710–718.

WRIGHT, F. S. (1971). Increasing magnitude of electrical potential along the renal distal tubule. *Am. J. Physiol.* **220**, 624–638.