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Proximal Tubular Reabsorption of Some Organic Acids in the Rat Kidney in vivo* **

By

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With 2 Figures in the Text

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Summary. Using a microperfusion technique proximal tubular reabsorption of sulfamerazine, phenobarbital, p-aminohippuric acid, uric acid, sulfaurea and N^4-acetylsulfamerazine was studied as a function of intratubular pH in the rat kidney in vivo. Transtubular permeabilities were related to in vitro lipoid solubility measurements and degrees of non-dissociation of the acids. It was found that

1. sulfamerazine and phenobarbital exhibit nonionic backdiffusion, the magnitude of which is dependent on lipoid solubility,
2. p-aminohippuric acid, uric acid and sulfaurea do not show this backdiffusion, a finding explained by lack of lipoid solubility,
3. N^4-acetylsulfamerazine, despite relatively high lipoid solubility, is not reabsorbed in the proximal tubule.

The classic concept of renal excretion of organic acids, consisting of glomerular filtration and unidirectional active secretion[17] has been challenged by the demonstration that clearance of certain weak organic acids is dependent on urinary pH[5,24] and on the pK_a of the acid[12]. MILNE et al.[14] therefore postulated the existence of a third mechanism involved in the excretion of these compounds, based on the observation of OVERTON[16] that molecules with high lipid/water partition coefficients are able in general to penetrate cell membranes more easily than those with low partition coefficients. Since the nonionized fraction of a weakly dissociating organic acid is more lipid soluble than the charged anion, the former can diffuse across the lipid-like barrier of the tubular cell. In acid urine, a gradient for the undissociated molecule from tubular fluid to plasma results in backdiffusion and decreased overall excretion; under

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conditions of alkaline urine the gradient is diminished or reversed, resulting in increased excretion of total compound. The magnitude of this effect is dependent on the degree of ionization and therefore pK_a of the acid.

This hypothesis has been utilized by MUDGE and WEINER[15] to explain different rates of excretion of different organic acids on the basis of a triphasic mechanism, consisting of glomerular filtration, tubular secretion, and non-ionic backdiffusion dependent on lipoid solubility. Not all of the evidence is in favour of this mechanism, however. In renal slice studies, DESPOPOULOS was unable to relate either uptake[8] or efflux[9] of weak organic acids to their pK_a or lipoid solubility. He questions the earlier conclusions on the basis that pH changes might have affected clearance values by altering the degree of binding to plasma protein[7].

Since the earlier techniques provide only indirect evidence on the mechanisms involved in renal handling of organic acids, the present study was undertaken in an attempt to determine directly 1. whether nonionic diffusion across tubular epithelium can occur, and 2. whether it is a component of renal excretion of actively transported compounds. Utilizing a newly-developed technique, sections of proximal tubule were perfused in vivo, and reabsorption of organic acids of differing pK_a and lipoid solubility was determined as a function of intratubular pH.

Methods

Experimental animals. Male albino rats (150—250 g) were maintained on a standard laboratory diet and received water ad lib. No food was given for 24 hours before the experiment. The animals were anesthetized by intraperitoneal injection of Inactin (90 mg/kg), the left kidney was exposed through a flank incision, freed from perirenal fat and immobilized in a plexiglas tray. A constant drip of prewarmed mineral oil onto the surface prevented cooling of the exposed kidney. Body temperature was kept constant on an electrically heated operating table. An intravenous infusion of Ringers solution (0.45 ml/hr) was administered throughout the course of the experiment.

Microperfusions. The apparatus and technique of microperfusion have been described in detail[21] and are elucidated only briefly here: Under microscopic observation a sharpened glass capillary pipette filled with colored mineral oil was inserted through the kidney capsule into a surface loop of proximal tubule. Small droplets of oil were injected into the tubular fluid stream to outline the course of the tubule. If at least two more loops appeared on the surface a second pipette, containing perfusion fluid and mounted on a microperfusion pump, was inserted into the more proximal loop. The lumen between the two puncture sites was then filled with mineral oil, preventing contamination of perfusate with glomerular filtrate. The oil-filled pipette was withdrawn, allowing filtrate to escape through the puncture, and, after reinsertion into the more distal loop, was used to collect samples of perfusate. Perfusion rate was kept constant at $20 \cdot 10^{-6}$ ml/min, a rate within the range of normal intratubular flow[18,22]. On terminating the experiment the perfused sections of proximal tubule were filled with liquid neoprene and their length determined by microdissection of the macerated kidney.

Perfusate consisted of a fluid containing sodium in a concentration found by GERTZ[10] to prevent net proximal transtubular flux of sodium and thereby of water, if isotonicity was maintained by a nonreabsorbable solute. No change in intratubular flow due to water reabsorption was therefore expected over the length of perfused section. This premise was tested by comparing inulin concentrations in samples of infused and withdrawn fluid, and was found to hold within the error of determinations. Perfusate was buffered to either pH 5 or pH 8 and had the following composition:

pH 5: NaCl 110 mM/l
Mannitol 35 mM/l
in phosphate buffer, consisting of 98.8 ml 0.022 M KH_2PO_4 and 1.2 ml 0.022 M Na_2HPO_4

pH 8: NaCl 68 mM/l
Mannitol 100 mM/l
in phosphate buffer, consisting of 5.5 ml 0.022 M KH_2PO_4 and 94.5 ml of 0.022 M Na_2HPO_4.

Measurement of freezing point depression of both solutions showed a range in osmolality from 300 to 310 mOsm/l; flame-photometric determination of sodium concentration ranged between 108 and 110 meg/l Na^+. Test substances were added in concentrations from 0.4 mM/l to 4.7 mM/l, thus leaving osmolalities virtually unchanged.

Chemical determinations. The proximal tubular reabsorption of the following organic acids was investigated:

a) sulfamerazine (2-sulfanilamido-4-methyl pyrimidine)
b) phenobarbital (5-phenyl-5-ethyl-barbituric acid)
c) PAH (p-aminohippuric acid)
d) uric acid
e) sulfaurea (1-(p-aminophenylsulfonyl)-urea)
f) N^4-acetylsulfamerazine.

Sulfonamide concentration in samples of fluid infused into and withdrawn from proximal tubule was determined by a micromodification[6] of the spectrophotometric method of BRATTON and MARSHALL[4]. PAH, phenobarbital and uric acid were obtained as the C^{14}-labelled compounds and radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer.

In vitro lipoid solubilities of the test substances at pH 5 and pH 8 were determined by shaking equal volumes of perfusate and lipoid solvent (toluene or chloroform) for 20 min, measuring the concentration of test substance in the aqueous phase before and after this procedure.

Results

Perfusion Experiments. On the basis of proximal reabsorption, the test substances can be divided into two main categories, the first including sulfamerazine and phenobarbital, the second including the remaining four acids.

Results of 37 perfusions at pH 5 and 22 perfusions at pH 8 with sulfamerazine-containing solutions, and 18 perfusions each with phenobarbital show a rapid decrease of intratubular concentration with perfused length, in some cases reaching the limit of detection within 1600 μ. No attempt was therefore made to perfuse longer sections of tubule. On

the assumption that the observed reabsorption was due to diffusion only, the percent decrease in concentration of both sulfamerazine (Fig. 1 a) and phenobarbital (Fig. 1 b) at pH 5 and pH 8 was plotted logarithmically against perfused length of tubule. Using linear regression analysis, straight-line relationships were obtained for the four sets of data. Correlation between the logarithm of absorption and perfused length was statistically highly significant ($p < 0.01$) in each case. Since the slope of

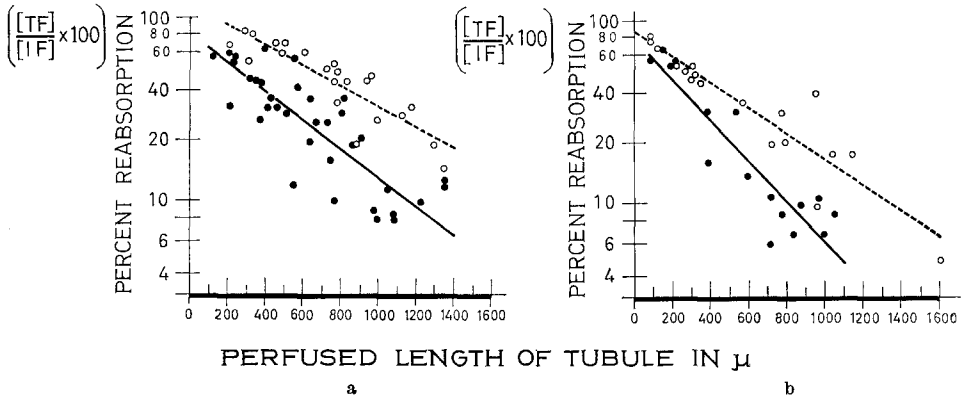

Fig. 1 a and b. Proximal tubular reabsorption of a sulfamerazine and b phenobarbital at pH 5 (•) and pH 8 (○) as a function of perfused length. *IF* concentration of testsubstance in perfusate; *TF* concentration in withdrawn sample

the lines is a relative measure of permeability it is evident from Fig. 1 that proximal epithelium is more permeable to both sulfamerazine and phenobarbital at pH 5 than at pH 8. In addition, relative permeabilities for phenobarbital are higher than the corresponding values for sulfamerazine.

In contrast to findings with sulfamerazine and phenobarbital, perfusions with PAH, uric acid, sulfaurea and N^4-acetylsulfamerazine show slight decrease in intratubular concentration of these compounds, the average value at 1600 μ being approximately 80% of control. The percent reabsorption was again plotted logarithmically as a function of perfused length (Fig. 2 a—d). The correlation coefficient for each regression line was statistically significant at or below the 5% level. In addition to the reduced reabsorption the slopes for PAH, uric acid and sulfaurea are steeper at pH 8. N^4-acetylsulfamerazine, although its rate of reabsorption is also low, has the steeper slope at pH 5.

Lipoid solubilities. Solubilities of the test substances in chloroform and toluene were related to degrees of ionization at pH 5 and pH 8 (Table). Transtubular permeabilities included in the table were calculated utilizing the equation derived in the appendix. Sulfamerazine and phenobarbital which are almost completely undissociated at pH 5 are

more soluble in both lipoid solvents at this value. Permeabilities also are higher than at pH 8. No significant lipoid solubilities were found for PAH, uric acid and sulfaurea, despite the relatively high proportion

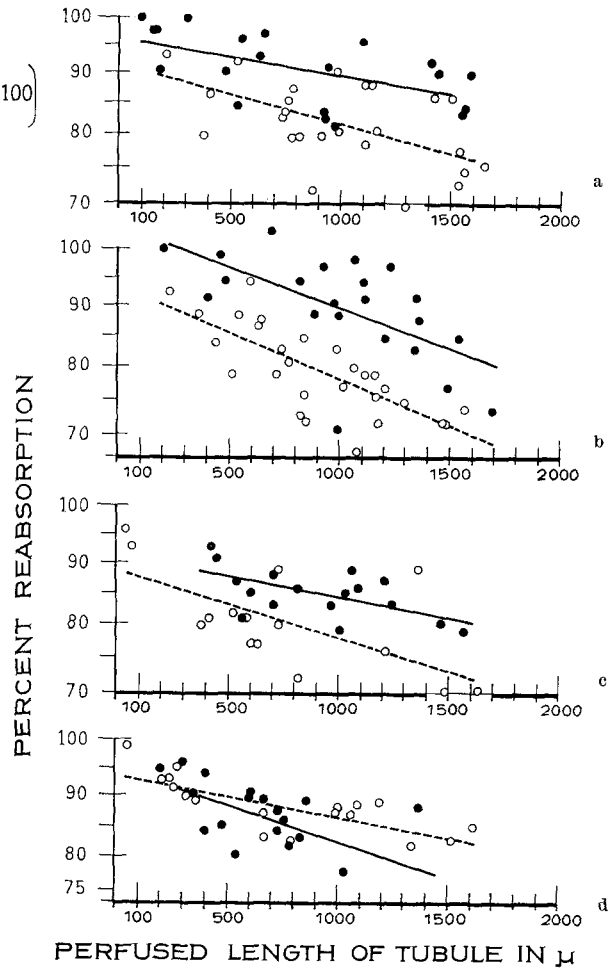

Fig. 2a—d. Proximal tubular reabsorption of a PAH, b uric acid, c sulfaurea and d N^4-acetylsulfamerazine at pH 5 (•) and pH 8 (○) as a function af perfused length. *IF* concentration of test-substance in perfusate; *TF* concentration in withdrawn sample. Note expanded scales on ordinates, compared to Fig. 1

at pH 5 of nonionized acid for the latter two compounds. Permeabilities of all three substances are correspondingly low. In contrast to sulfamerazine and phenobarbital values are slightly higher at pH 8. The lipoid solubility of N^4-acetylsulfamerazine at pH 5 is comparable to that of the parent compound, as is the undissociated fraction of the acid. However,

Table. *Comparison of physical constants to in vivo permeability*

Relative lipoid solubilities are expressed on the basis of concentration measurements in perfusate before (c_i) and after (c_e) equilibration with chloroform or toluene

	Sulfamerazine		Phenobarbital		PAH		Uric Acid		Sulfaurea		N^4-acetylsulfamerazine	
pK_a	7.0		7.2		3.8		5.8		5.6		6.6	
pH	5	8	5	8	5	8	5	8	5	8	5	8
percent non-ionized	99	9	99	14	6	0	86	1	80	0	98	4
lipoid solubility $\left(100-\frac{100c_e}{c_i}\right)$ chloroform	72	34	83	56	2	2	4	2	0	0	42	0
lipoid solubility $\left(100-\frac{100c_e}{c_i}\right)$ toluene	8	2	37	12	1	1	0	1	2	2	8	0
permeability (mm/sec · 10–4)	96	68	167	85	4	6	8	10	4	7	8	4

transtubular permeabilities are of the same order of magnitude as those of the nonlipoid soluble compounds. Interestingly, the higher permeability was found at pH 5.

Discussion

The results of experiments with sulfamerazine and phenobarbital show good qualitative agreement between concentration of non-dissociated acid and magnitude of lipoid solubility and transtubular permeability as a function of H^+ concentration. pH-dependent renal excretion of sulfamerazine[2] and phenobarbital[23] had previously been shown in clearance studies in the dog. Substrate to creatinine clearance ratios were low in both cases and for phenobarbital were shown to be unaffected by administration of probenecid. It seems clear, therefore, that the mechanism of excretion of these two compounds consists of glomerular filtration and subsequent non-ionic backdiffusion.

PAH, uric acid and sulfaurea, on the other hand, do not demonstrate this backdiffusion. Their low rates of reabsorption were slightly higher in the alkaline medium and were not dependent on the ratio of ionized to nonionized acid. Since both increase and decrease of pH may affect cell permeability[11],

it is likely that the perfusates employed changed the characteristics of the tubular wall, increasing its permeability to all substances. This effect seems more pronounced at the higher pH value. Perfusions with unbuffered solutions containing PAH showed no reabsorption of this substance[6]. The assumption of an unspecific pH effect is borne out by the finding that uric acid, with the lowest molecular size of the three substances, has the highest permeability. The insignificant reabsorption of these compounds, corresponding to a lack of solubility in lipoid solvents, further strengthens the hypothesis that backdiffusion takes place predominately through a lipidlike cellular phase.

Although the results demonstrate the existence of nonionic diffusion, no evidence was found to support the hypothesis of MUDGE and WEINER[15] that this mechanism plays a part in renal elimination of actively secreted organic acids.

These authors assume equal secretory rates for PAH and other organic acids which are dissociated in the physiological pH range. Differences in overall excretion could be due to differences in the degree of backdiffusion. Sulfaurea, with a structure similar to that of PAH, possesses the dissociable hydrogen ion given as the only requirement for active transport. In the absence of both lipoid solubility and non-ionic diffusion, as determined in the present experiments, a high rate of excretion would be expected. Clearance of this compound, however, is low[13]; this is a result not predicted by the hypothesis.

The findings with N^4-acetylsulfamerazine, which is actively secreted by the kidney[3], also are incompatible with the MUDGE hypothesis. Although the lipoid solubility of the nonionic form is only slightly less than that of the parent compound, no transtubular permeability comparable to that of sulfamerazine was found. It might be argued that the lower pK_a and the insolubility of the ionized form of N^4-acetylsulfamerazine would tend to enhance the effect of a possible change toward neutrality in tubules perfused at pH 5. In this case a high rate of reabsorption would be expected in the initial tubular segment, in which buffering capacity of the perfusate is largest. Inspection of Fig. 2d does not show such an effect, however. A second explanation of the lack of backflux is that the acid does indeed penetrate the cellular barrier by nonionic diffusion, but is transported back into the lumen via the active secretory mechanism. The finding of a slightly higher permeability at pH 5 compared to that at pH 8 is in agreement with this conjecture, since some escape of nonionized acid into the bloodstream might be expected. However, recent perfusion experiments on distal tubule[1] also failed to provide evidence for nonionic diffusion of N^4-acetylsulfamerazine. In the absence of an active transport mechanism for organic acids in this nephron segment, the conclusion remains that N^4-acetylsulfamerazine,

despite its in vitro lipoid solubility, is not reabsorbed via nonionic diffusion through the lipid-like barrier of the tubular membrane.

Appendix

Using Fick's laws of diffusion the axial flow Φ_1 of substrate at any point x of a perfused tubule can be expressed by the equation

$$\Phi_1 (x) = v_1 \cdot c_1 (x), \tag{1}$$

in which v_1 represents linear flow velocity and $c_1 (x)$ concentration of substrate at point x.

The transtubular flux $\Phi_{1,2}$ at this point is given by

$$\Phi_{1,2}(x) = P_{1,2} [c_1(x) - c_2(x)], \tag{2}$$

in which $P_{1,2}$ is the transtubular permeability for substrate and c_2 the peritubular concentration.

Summation of the two fluxes results in

$$- \pi r_1^2 \cdot \Phi_1(x) = 2 \pi r \int \Phi_{1,2}(x)\, dx$$

or

$$- \frac{d\Phi_1 (x)}{dx} = \frac{2}{r_1} \Phi_{1,2} (x), \tag{3}$$

where r_1 represents the radius of the tubule.

Since choice of the perfusate was such that v_1 remained unchanged over the perfused length of tubule, equation (1) may be substituted into equation (3) as follows:

$$- \frac{dc_1 (x)}{dx} = \frac{2}{v_1 r_1} \Phi_{1,2} (x). \tag{4}$$

Substitution of equation (2) into equation (4) then results in

$$- \frac{dc_1 (x)}{dx} = \frac{2}{v_1 r_1} P_{1,2} [c_1(x) - c_2(x)]. \tag{5}$$

Under the assumption that peritubular blood flow is sufficient to remove completely the relatively small amounts of substrate diffusing through the wall of the perfused tubule, the peritubular concentration of substrate is negligibly low. On integration equation (5) therefore reduces to

$$c_1(x) = K \cdot e^{- \frac{2 P_{1,2}}{v_1 r_1} \cdot x} \tag{6}$$

where K is the constant of integration.

At the perfusion site where x equals zero, the initial concentration c_1 of substrate equals

$$c_1 = K \cdot e^{- \frac{2 P_{1,2}}{v_1 r_1} \cdot 0},$$

and therefore

$$K = c_i,$$

so that equation (6) becomes

$$c_1(x) = c_i \, e^{-\frac{2P_{1,2}}{v_1 r_1} \cdot x}. \tag{7}$$

Transformation of equation (7) results in

$$P_{1.2} = \frac{v_1 r_1}{2x} \cdot \ln \frac{c_i}{c_1(x)}. \tag{8}$$

Since r_1 is known[22] and v_1 can be obtained from perfusion rate, measurement of perfused length of tubule and initial and final substrate concentrations allows solution of equation (8).

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