Renal secretion of diphosphonates in rats

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Renal secretion of diphosphonates in rats. Diphosphonates, characterized by a P—C—P bond, are relatively new experimental drugs used for the treatment of myositis ossificans, dental calculus, nephrolithiasis and Paget's disease. These compounds are not metabolized and the fraction which is not taken up by the skeleton is excreted unchanged in the urine. In the present study, the renal clearances of two ^{14}C-labelled diphosphonates, disodium ethane-1-hydroxy-1,1-diphosphonate (C_{EHDP}) and disodium dichloromethylene diphosphonate (C_{Cl_2MDP}) have been measured in conscious rats. The clearances have been found to be higher than the glomerular filtration rate (GFR), $C_{diphosphonate}/$ GFR being about 1.5. This observation indicates net tubular secretion of both drugs. High plasma concentration of EHDP or Cl_2MDP significantly depressed C_{EHDP}, whereas C_{EHDP} was not influenced by varying urine pH, by infusing NH_4Cl or $NaHCO_3$, or by simultaneous administration of high doses of para-aminohippurate (PAH), probenecid, N-methylnicotinamide or Ca-EDTA. High plasma concentration of inorganic phosphate depressed C_{EHDP} and also depressed the *in vitro* ultrafiltrability of EHDP. In conclusion, these results provide evidence of an active renal transport of diphosphonates which appears distinct from the mechanisms handling organic acids, organic bases and EDTA in the rat kidney.

Secrétion renale des diphosphonates chez le rat. Les diphosphonates, caractérisés une liaison P—C—P, sont des médicaments relativement nouvaux utilisés dans le traitement de la myosite ossifiante, des calculs dentaires, de la lithiase urinaire et de la maladie de Paget. Ces substances ne sont pas métabolisées et la fraction qui n'est pas captée par le squelette est excrétée sans modifications dans l'urine. Dans ce travail les clearances rénales de deux diphosphonates marqués par le ^{14}C, disodium éthane-1-hydroxy-1,1-diphosphonate (C_{EHDP}) et disodium dichlorométhylène diphosphonate (C_{Cl_2MDP}) ont été mesurées chez des rats conscients. Ces clearances sont supérieures au débit de filtration glomérulaire (GFR), le rapport $C_{diphosphonate}/$GFR est d'environ 1,5. Cette observation indique une secrétion tubulaire nette des deux drogues. Des concentrations plasmatiques élevées de EHDP ou de Cl_2MDP dépriment significativement C_{EHDP} alors que C_{EHDP} n'est pas influencé par les variations du pH de l'urine, par la perfusion de NH_4Cl ou de $NAHCO_3$, ou par l'administration simultanée de doses élevées de PAH, de probénécide, de N-methyl-nicotamide ou de Ca-EDTA. Des concentrations plasmatiques élevées de phosphate inorganique abaissent C_{EHDP} et diminuent aussi l'ultrafiltrabilité *in vitro* de EHDP. En conclusion, ces résultats apportent la preuve d'un transport rénal actif des diphosphonates qui paraît distinct des mécanismes qui assurent le transport des acides organiques, des bases organiques et de l'EDTA dans le rein de rat.

Diphosphonates are compounds characterized by a P—C—P bond. They have recently been introduced in the treatment of diseases of calcium metabolism. The rationale lies in their marked inhibitory effect on hydroxyapatite crystal growth [1, 2] and dissolution [3, 4] *in vitro*, and their inhibition of calcium phosphate deposition [5] and resorption [3, 4] *in vivo*. One diphosphonate, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP), has been used successfully in slowing down or preventing the progress of calcification in myositis ossificans [6, 7] and calcinosis universalis [8]. It also reduces dental calculus formation [9, 10]. EHDP has been found also to decrease bone turnover and improve symptoms in patients suffering from Paget's disease [11, 12].

The bioavailablity and disposition of EHDP have been studied in four species by Michael, King and Wakim [13]. They found that EHDP was not appreciably metabolized in the rat. Approximatively one-half of an absorbed dose went into bone and the remaining fraction was excreted unchanged in the urine. Only minute amounts were taken up by soft tissues. Thus, kidney and bone seemed to play a major role in the *in vivo* fate of EHDP.

The present work was designed to study in conscious rats the renal handling of two ^{14}C-labelled diphosphonates, EHDP and dichloromethylene diphosphonate (Cl_2MDP) by clearance technique. Since both diphosphonates were found to be secreted by the kidney, an attempt was made to characterize the mechanisms involved. The effect of change of urinary pH and of several agents known to be secreted by the renal tubule on the clearance of EHDP was investigated. The results of these experiments suggest that the secretory mechanism of diphosphonates differs from those already described for organic acids, bases and EDTA.

Methods

Experimental design. All experiments were performed on male Wistar rats from our own breeding colony. Prior to the experimental day, the animals were fed a commercial diet (Altromin 1314) and had free access to water.

To obtain steady rates of urine flow, the dead space of the urinary tract was diminished by surgical removal of the dome of the urinary bladder while the rats were lightly anesthetized with ether, 18 to 24 hr prior to the experiment. On the experimental day, the rats were weighed and put into restrictive cages specially designed for studying renal function in conscious rats according to a method previously described [14]. Urine was collected anaerobically in tared test tubes containing 1 ml of Xylol and blood samples were taken from a hind limb vein.

For infusions, a lateral vein was catheterized. Initially, a dose (priming) of inulin (80 mg/kg of body wt) dissolved in 0.15M NaCl was injected i.v. in a volume of 2.5 ml/kg of body wt. Solutions containing various concentrations of ^{14}C-labelled diphosphonates dissolved in 0.15M NaCl were then infused by means of a micropump (Ismatec) with a delivery rate of 3.9 to 4.0 ml/hr. In some experiments the infusion solution also contained either 120 mM NH_4Cl, $NaHCO_3$ or 150 mM inorganic phosphate (P_i), respectively. In other experiments, *p*-aminohippuric acid (PAH), probenecid, N-methylnicotinamide (NMN) or ethylenediaminetetraacetic acid (EDTA) was added to both the infusion and the priming solution. The diphosphonates and P_i were not added to the priming solution in order to avoid the possibility of a local precipitation at the site of the i.v. injection through the formation of polynuclear complexes of EHDP (see following). The pH of all solutions was adjusted to 7.4 and the osmolality was adjusted to 300 to 310 mOsm/liter where necessary by additon of NaCl. For the P_i solution, pH 7.4 was obtained by combining Na_2HPO_4 and NaH_2PO_4 in adequate proportions. Inulin was infused at 40 mg/rat hr in all experiments, while the radioactivity of the diphosphonate infused varied from 272 to 1064 nCi/rat hr.

After 120 min of equilibration, urine was collected for two periods (I, II) of 30 to 45 min each. Blood samples were taken before the first urine period (1), between the first and the second (2) and at the end of the second urine collection period (3). Urine volume (V), the clearance of inulin (C_{In}) as a measure of glomerular filtration rate (GFR) and the clearance of the respective diphosphonate (C_{EHDP}, C_{Cl_2MDP}) were calculated for each urine period using a standard formula. The blood concentrations were the arith-metical mean of samples 1 and 2 for urine I and of 2 and 3 for urine II.

Analytical methods. Urinary volume (V) was determined by weighing.

Inulin was determined in plasma and urine by the anthrone method [15]. In preliminary experiments, inulin was also determined by the diphenylamine method [16] in order to rule out any interference of the diphosphonate with the anthrone reagent. The ratio C_{In} anthrone/C_{In} diphenylamine did not differ significantly from 1.0. A possible interference of EHDP in the anthrone method for assaying inulin in urine was also tested *in vitro* by adding cold EHDP to urine samples of known inulin concentration. No interference was found.

^{14}C-labelled diphosphonate activity in urine, plasma ultrafiltrates or plasma was analyzed by adding urine or plasma ultrafiltrate (20 μl), or plasma (25 to 50 μl), to vials containing liquid scintillation solution (10 ml). The composition of the scintillation fluid was toluene, 600 ml; ethylene-glycolomonoethylether, 400 ml; naphthalene, 80 g; and Butyl-PBD (Ciba-Geigy), 7.0 g. Duplicate samples were counted in a scintillation spectrometer (Packard Tricarb, Model 3950). No quenching was observed when plasma or urine was added in the volumes stated above. Since Michael et al [13] had found that ^{14}C-EHDP was not appreciably metabolized in the rat, the radioactivity measured was considered to be entirely unmetabolized EHDP.

The ultrafiltrable fraction of diphosphonates added *in vivo* and *in vitro* to plasma was measured as follows: *a) In vivo addition:* Rats were anesthetized with ether after a 150-min infusion period and aortic blood was obtained while the infusion continued. An Amicon cell (Model 12, Amicon Corp., Lexington, MA) fitted with either an XM-50 or an XM-100A Diaflo filter (Amicon Corp., Lexington, MA) was operated at a gas pressure of 4 atm (1% CO_2 and 99% air) to ultrafilter the plasma. Subsequently, plasma before ultrafiltration and aliquots of ultrafiltrate were analyzed for ^{14}C-EHDP, and the concentrations of phosphate and protein were also determined. The concentration in the ultrafiltrate was expressed as percent of the plasma concentration. *b) In vitro addition:* Ultrafiltrability was likewise determined after addition of ^{14}C-EHDP or ^{14}C-Cl_2MDP to plasma *in vitro.*

Equilibrium dialysis was used to determine the dialyzable fraction of ^{14}C-EHDP. A volume of 2.5 ml was placed into each of two plastic dialysis cells, separated by a Technicon Cuprophan 105–1058 membrane. The cells were rotated at 130 rpm in a 37°C incubator whose atmosphere was saturated with 5% CO_2. Plasma was dialyzed against HEPES (N-2-

hydroxyethylpiperazine-N'-2-ethane sulfonic acid)-Krebs-Ringer buffer (HEPES, 50 mM; pH 7.4), and equilibration was reached after 75 min. Correction was made for water movements by estimating plasma protein concentration according to the method of Lowry et al [17].

Inorganic phosphorus was determined in urine, plasma ultrafiltrates and plasma as described by Bisaz, Russell and Fleisch [18]. Calcium concentrations were estimated by atomic absorption spectroscopy (Perkin Elmer, Model 290 B). Osmolality of infusion solutions was measured with an osmometer (Advanced, Model 3W, Advanced Inc., Needham Heights, MA).

Materials. The radioactive diphosphonates used were as follows: Na_2H_2EHDP-^{14}C, sp. activ. 6.1 mCi/g and $Na_2H_2Cl_2MDP$-^{14}C sp. activ. 1.6 mCi/g. The chemical purity of both diphosphonates was greater than 99%, as assessed by reverse isotope dilution, thin-layer chromatography, nuclear magnetic resonance and X-ray diffraction [13]. All chemicals were of analytical grade.

Statistical analysis. The experimental results are expressed as mean ± SEM. Significance of the difference between groups was determined by Student's t test.

Fig. 1. $C_{diphosphonate}$ and C_{In}: Fig. 1A, EHDP; and Fig. 1B, Cl_2MDP. Following an equilibration period of 120 min, two clearance periods (I, II) of 30 min each were performed as indicated in Methods. The two periods are represented separately. The infused amount of diphosphonates was 2.14 μmoles/rat hr in both groups.

Results

The clearances of the two diphosphonates (C_{EHDP}, C_{Cl_2MDP}) infused at a level of 2.14 μmoles/rat hr and also of inulin (C_{In}) are depicted in Fig. 1. As shown in Fig. 1A, the values of C_{EHDP}, i.e., 12.25 ± 0.53 in period I and 11.62 ± 0.44 ml/kg·min ($N = 14$) in period II, exceeded C_{In} which was 9.38 ± 0.50 and 8.70 ± 0.36 ml/kg·min, respectively. The fractional excretion of EHDP represented by the ratio C_{EHDP}/C_{In} was 1.32 ± 0.05 and 1.34 ± 0.04 for periods I and II, respectively. Similar results were obtained with Cl_2MDP. Indeed, Fig. 1B shows that C_{Cl_2MDP} was 12.84 ± 1.32 in period I and 12.79 ± 1.74 ml/kg·min ($N = 6$) in period II, whereas C_{In} was 8.80 ± 0.84 and 7.83 ± 0.57 ml/kg·min for the same periods. The fractional excretion of Cl_2MDP (C_{Cl_2MDP}/C_{In}) was 1.46 ± 0.06 and 1.53 ± 0.13 for periods I and II, respectively. These results demonstrate a net tubular secretion of both EHDP and Cl_2MDP. They were obtained under steady-state conditions as indicated by the nonsignificant differences between period I and period II for the respective clearances. About 50% of the infused diphosphonates was excreted in the urine.

It is known that the clearance of a substance secreted by a system exhibiting maximal transport rate decreases when its plasma concentration increases. Clearance data obtained when plasma concentrations of EHDP were altered by infusing doses from 272
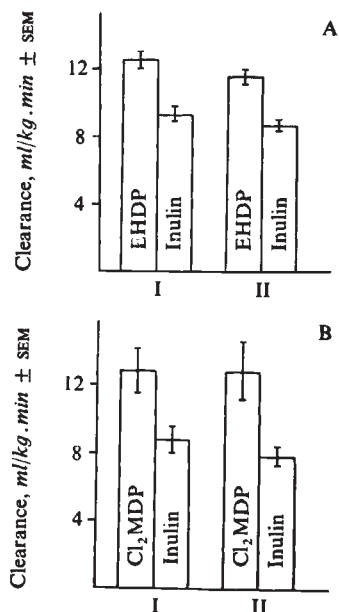
(group A) to 3400 nmoles/rat hr (group E) are summarized in Table 1. Increasing the plasma concentration of EHDP ([EHDP]$_{Pl}$) from 0.72 to 3.79 μM (groups A, B and C) did not significantly change C_{EHDP} or the ratio C_{EHDP}/C_{In}. However, at higher [EHDP]$_{Pl}$ (groups D and E), C_{EHDP} and C_{EHDP}/C_{In} decreased significantly when compared to the values observed at low [EHDP]$_{Pl}$ (group A).

Fig 2 illustrates the inverse relationship between [EHDP]$_{Pl}$ and C_{EHDP}/C_{In} ($r = -0.53$, $P < 0.01$, $N = 47$), when the individual values of all experiments are represented. As previously indicated in Table 1, the fall of C_{EHDP} when [EHDP]$_{Pl}$ increases was not due to alterations of C_{In} as the relationship between [EHDP]$_{Pl}$ and C_{EHDP}/C_{In} shows the same pattern (Fig. 2). Fig. 2 also demonstrates that all rats but one had a C_{EHDP}/C_{In} ratio above that of unity. When EHDP was increased 6.5 times over that of group E, [EHDP]$_{Pl}$ rose continuously during the experiment from 500 to 1000 μM. On the assumption that this increase was a linear function of time, C_{EHDP} and C_{EHDP}/C_{In} were calculated and very low values were obtained. Indeed, C_{EDHP} was 1.30 ± 0.32 ml/kg·min (period I) and 1.04 ± 0.32 ml/kg·min (period II, $N = 5$). C_{EHDP}/C_{In} was 0.16 ± 0.04 ml/kg·min (period I) and 0.12 ± 0.04 ml/kg·min (period II) since C_{In} remained unchanged at 8.33 ± 0.17 ml/kg·min (period I) and 8.26 ± 0.35 ml/kg·min

Table 1. Clearance of EHDP in conscious rats[a]

Group	N	Dose of EHDP $nmoles/rat\ hr$	$[EHDP]_{Pl}$ μM	C_{EHDP} $ml/kg \cdot min$	C_{In} $ml/kg \cdot min$	C_{EHDP}/C_{In}
A	9	272	0.72 ± 0.03	14.22 ± 0.85	8.21 ± 0.30	1.74 ± 0.10
B	6	544	1.68 ± 0.05	13.03 ± 0.69	8.74 ± 0.41	1.51 ± 0.09
C	9	1072	3.79 ± 0.08	13.52 ± 0.62	8.39 ± 0.21	1.61 ± 0.06
D	14	2144	7.30 ± 0.18	11.94 ± 0.34[b]	9.05 ± 0.34	1.34 ± 0.04[d]
E	8	3400	13.05 ± 0.29	10.33 ± 0.41[c]	7.44 ± 0.27	1.39 ± 0.03[c]

[a] All values are mean $\pm$ SEM. C_{EHDP}=clearance of disodium ethane-1-hydroxy-1,1-diphosphonate; C_{In}= clearance of inulin; $[EHDP]_{Pl}$=concentration of EHDP in plasma. N=number of rats. For each rat, the average of the three $[EHDP]_{Pl}$ values and the average of the two clearance periods were used for the calculation of the mean of each group.
[b] $P < 0.05$ as compared to group A.
[c] $P < 0.01$ as compared to group A.
[d] $P < 0.001$ as compared to group A.

(period II). These results could be explained by the formation of polynuclear complexes with calcium in this concentration range of EHDP as suggested by the work of Grabenstetter and Cilley [19]. Presumably, these large molecular weight complexes would not be filtered by the kidney glomeruli. For this reason, the ultrafiltrable fraction of EHDP was not determined in this condition and the results were not plotted in Fig. 2.

Following the infusion of EHDP, the ultrafiltrable fraction of groups A, C and E, as described in Table 1, was measured using a Diaflo XM 50 filter. Group A

Fig. 2. C_{EHDP}/C_{In} *related to plasma concentration of the drug.* Each *point* represents the mean of two clearance periods in one rat.

was $77 \pm 1\%$ ($N=4$); group C, $63 \pm 2\%$ ($N=5$); and group E, $67 \pm 5\%$ ($N=3$). When the diphosphonates were added to plasma *in vitro* in order to obtain a concentration of 5 μM, the ultrafiltrable fraction of EHDP was $64 \pm 4\%$ ($N=4$), and that of Cl_2MDP was $83 \pm 9\%$ ($N=3$) with the XM 50 filter (mol wt cutoff of about 50,000). It was $75 \pm 4\%$ ($N=3$) for EHDP and $95 \pm 5\%$ ($N=3$) for Cl_2MDP with an XM 100 A filter (mol wt cutoff of about 100,000).

In two determinations, the dialyzable fraction of EHDP was 81.5 and 77.2% ($[EHDP]_{Pl}=1$ μM) and 74.9 and 78.6% ($[EHDP]_{Pl}=100$ μM). These results correspond approximately to the values of the ultra-fitrable fraction of EHDP indicated above.

The excretion rate of certain organic acids depends upon urinary pH, the excretion being higher in alkaline than in acid urine [20]. Data from clearances measured in animals perfused with 480 $\mu moles/rat$ hr of either $NaHCO_3$ or NH_4Cl concomitantly with EHDP at a dose of 1.07 $\mu moles/rat$ hr are given in Table 2. C_{EDHP}/C_{In} was not significantly different in alkalinized compared to acidified rats. In this latter group, C_{EHDP} and C_{In} were slightly lower than in the group infused with $NaHCO_3$.

The influence of substances secreted by the system transporting organic acids such as PAH and probenecid and by the system secreting organic bases (e.g., NMN) on the clearance of EHDP is summarized in Table 3. PAH up to a dose ratio of 820 (EHDP=1) probenecid and NMN up to a dose ratio of 100 (EHDP=1) did not affect the C_{EHDP}/C_{In} ratio. EDTA is a substance reported to be secreted without being influenced by changes in urinary pH and also independently of substances of the organic acid and base group [21]. EDTA, when given as a calcium complex at a dose ratio of 31 (EHDP=1), did not influence C_{EHDP}. Total plasma calcium concentration was the
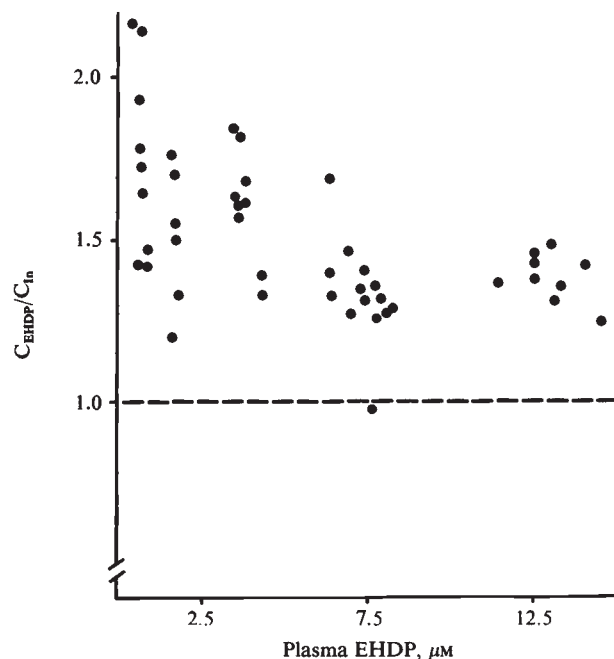
Table 2. Effect of systemic acidification and alkalinization on the renal handling of EHDP in rats[a]

Observation	NaHCO$_3$ ($N=9$)	NH$_4$Cl ($N=9$)
Urine pH	7.91 ± 0.05	6.10 ± 0.06[b]
C$_{EHDP}$	11.03 ± 0.73	9.35 ± 0.77
C$_{In}$	8.48 ± 0.47	7.04 ± 0.33[c]
C$_{EHDP}$/C$_{In}$	1.30 ± 0.06	1.36 ± 0.13

[a] All values are mean $\pm$ SEM. C$_{EHDP}$=clearance of disodium ethane-1-hydroxy-1,1-diphosphonate; C$_{In}$= clearance of inulin. N=number of rats. For each rat the average of the two clearance periods was used for the calculation of the mean of the group.
[b] $P < 0.001$ as compared to the alkalinized group.
[c] $P < 0.05$ as compared to the alkalinized group.

Fig. 3. *Influence of Cl$_2$MDP-loading on C$_{EHDP}$.* Total dose of EHDP per experiment was 2.45 μmoles in both control and experimental ($+$Cl$_2$MDP) groups. The latter received in addition a total dose of Cl$_2$MDP of 76 μmoles ("priming" 4 μmoles/rat $+$ infusion 72 μmoles/rat). Dose ratio was 31:1 (EHDP=1). Number of rats: control, 6; Cl$_2$MDP, 5. Clearances were calculated as explained in legend to table 2. **$P < 0.001$.

same in both groups (EDTA, 10.16 ± 0.13 mg/100 ml, $N=4$; control, 10.18 ± 0.8 mg/100 ml, $N=4$).

As shown in Fig. 1B, Cl$_2$MDP, the other diphosphonate, is also secreted. At a dose ratio of 31 (EHDP=1), equal to that used with EDTA, Cl$_2$MDP did significantly decrease C$_{EHDP}$ without altering C$_{In}$ (Fig. 3).

Inorganic phosphate (P$_i$) is another substance for which evidence of tubular secretion in the rat nephron has been presented very recently [22]. Results concerning the influence of i.v. loading of P$_i$ on renal handling of EHDP are summarized on Table 4. The data of the control group show that stable plasma concentrations and clearance values of EHDP and P$_i$ were reached in two clearance periods (I, II) measured after two hours' equilibration. In the P$_i$-loaded group, [EHDP]$_{Pl}$ was significantly higher in all three blood samples (1, 2 and 3) and steady-state concentrations were not achieved. Assuming that the fall of [EHDP]$_{Pl}$ was a linear function of time, which seems likely from the data presented, clearance calculations show that C$_{EHDP}$ was
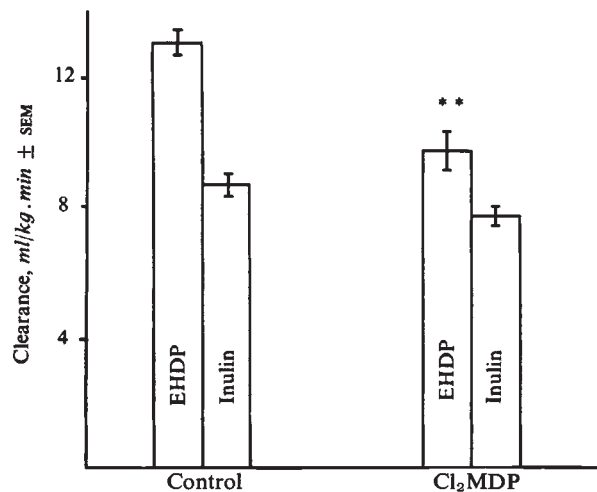
significantly lower in P$_i$-loaded animals as compared to the controls. As C$_{In}$ was the same in both groups, the fractional excretion of EHDP was significantly diminished in the P$_i$-loaded group ($P < 0.001$) and this effect was most striking in period I where it fell significantly below unity ($P < 0.02$). Note that the renal handling of P$_i$ remained constant during periods I and II in both groups.

In another similar experiment, the ultrafiltrability (XM 50 Diaflo ultrafilter) of EHDP and P$_i$ was measured at the end of a clearance period in the absence and presence of P$_i$ loading. In the absence of P$_i$ loading, C$_{EHDP}$/C$_{In}$ was 1.96 ± 0.20 ml/kg·min and the ultrafiltrable fraction of EHDP was $63 \pm 2\%$ ($N=5$).

Table 3. Effect of various substances secreted in rat kidney on C$_{EHDP}$/C$_{In}$ ratio in rats[a]

Substance and total dose per 1 experiment, μmoles		EHDP total dose per 1 experiment, μmoles	Dose ratio (EHDP=1)	Treated C$_{EHDP}$/C$_{In}$	Control C$_{EHDP}$/C$_{In}$
PAH	925[b]	9.7	95	1.28 ± 0.05 ($N=5$)	1.29 ± 0.03 ($N=4$)
	1000[c]	1.2	820	1.83 ± 0.07 ($N=4$)	1.84 ± 0.06 ($N=4$)
Probenecid	124[d]	10.0	12	1.35 ± 0.17 ($N=3$)	1.23 ± 0.07 ($N=4$)
	124[d]	1.2	100	2.40 ± 0.15 ($N=4$)	2.08 ± 0.08 ($N=4$)
NMN	129[e]	1.2	105	1.62 ± 0.06 ($N=6$)	1.55 ± 0.15 ($N=5$)
Ca-EDTA	88.5[f]	2.5	36	1.44 ± 0.07 ($N=5$)	1.38 ± 0.13 ($N=5$)

[a] Substances were administered in 0.15 M NaCl solution as follows: *PAH:* [b] "priming" 25 μmoles/rat $+$ infusion 900 μmoles/rat; [c] "priming" 75 μmoles/rat $+$ infusion 925 μmoles/rat. *Probenecid:* [d] "priming" 100 μmoles/rat $+$ infusion 24 μmoles/rat. *NMN:* [e] "priming" 7 μmoles/rat $+$ infusion 122 μmoles/rat. *Ca-EDTA:* [f] "priming" 15 μmoles/rat $+$ infusion 73.5 μmoles/rat. All values are mean $\pm$ SEM. N=number of rats. For each rat the average of clearance periods was used for the calculation of the mean of the group.

Table 4. Influence of an infusion of inorganic phosphate (P_i) on the renal handling of EHDP in rats[a]

Group	Control (N=9)			P_i-loaded (N=9)		
Clearance period	I	II		I	II	
Plasma sample	1	2	3	1	2	3
Plasma-EHDP, μM	3.68±0.15	3.67±0.16	3.90±0.10	9.96±1.14[b]	8.05±0.97[c]	5.42±0.68[d]
Plasma-P_i, mM	2.69±0.10	2.67±0.08	2.55±0.09	5.63±0.09[b]	5.78±0.13[b]	6.14±0.24[b]
Plasma-Ca, mM		2.23±0.12			1.59±0.17	
C_{In}, ml/kg·min		8.39±0.16	8.37±0.28		8.41±0.20	8.52±0.24
C_{EHDP}, ml/kg·min		13.87±0.61	13.16±0.65		5.70±0.83[b]	8.42±1.03[c]
C_{EHDP}/C_{In}		1.65±0.07	1.57±0.05		0.68±0.10[b]	1.00±0.13[b]
C_{P_i}, ml/kg·min		1.38±0.09	1.35±0.17		8.39±0.16[b]	8.63±0.29[b]
C_{P_i}/C_{In}		0.16±0.01	0.16±0.02		1.00±0.01[b]	1.02±0.04[b]

[a] All values are mean±SEM. Both control and P_i-loaded animals received EHDP i.v. at a dose of 18 nmoles/min. The P_i-loaded rats were given P_i at a dose of 10 μmoles/min. For the P_i solution, pH 7.4 was obtained by combining Na_2HPO_4 and NaH_2PO_4 in adequate proportion.
[b] $P < 0.001$ as compared to the respective control value.
[c] $P < 0.01$ as compared to the respective control value.
[d] $P < 0.05$ as compared to the respective control value.

In the P_i-loaded group, C_{EHDP}/C_{In} was 0.82±0.18 ml/kg·min but the ultrafiltrable fraction of EHDP was markedly reduced at 28±8% (N=3). No significant difference was observed between the two groups with respect to the ultrafiltrability of P_i (unloaded: 95±2%, N=5; P_i-loaded: 91±3%, N=3).

Discussion

The data presented in Figs. 1 and 2 and Table 1 indicate that the diphosphonates EHDP and Cl_2MDP are eliminated in the urine not only by glomerular filtration but also by tubular secretion. Even if an error of 10% in the clearance procedure were to occur, only one out of 47 animals studied would display a C_{EHDP}/C_{In} ratio below that of unity (Fig. 2). In addition, studies using ultrafiltration and dialysis techniques suggest an incomplete ultrafiltrability of both diphosphonates. The extrapolation of these results obtained *in vitro* to the actual glomerular filtration fraction of either diphosphonate suggests that the relative importance of the secretory process is, in fact, underestimated, when only the ratio $C_{diphosphonate}/C_{In}$ is considered. But as it is uncertain to what extent the ultrafiltrability determined *in vitro* may reflect the *in vivo* process, no "corrective" calculations were attempted.

The trend of C_{EHDP} and C_{EHDP}/C_{In} to decrease at plasma concentrations above 7.5 μM (Table 1, Fig. 2) suggests that the secretory process for EHDP might be saturable. However, in spite of a dialyzable fraction of EHDP which appears similar at either 1 or 100 μM, this slight reduction in C_{EHDP} could be due to a small change in the glomerular ultrafiltrability which is

difficult to assess *in vivo* and also may not be detected by *in vitro* studies.

The marked fall of C_{EHDP} at [EHDP]$_{Pl}$ higher than 400 μM is very likely due to the formation of polynuclear complexes of EHDP with calcium as described by Grabenstetter et al [19].

The net renal secretion of diphosphonates suggests an active renal cellular uptake of these drugs. Support for this concept comes also from incubation of rat renal cortical slices with ^{14}C-EHDP *in vitro* [23].

In an attempt to characterize the mechanism of this tubular secretion, two types of experiments were initiated:

First, the influence of a variable urinary pH on the excretion of EHDP was examined. Other investigations have described four pKa values for EHDP, namely, 11.16; 7.00 and 2.80 [24]; and 1.7 [25]. In an infusion solution, at pH 7.4, therefore, EHDP should be present as an equilibrium mixture of the anionic species (mainly bivalent and trivalent, the nonionic fraction being negligible). However, the state of EHDP in plasma is very difficult to predict, since besides a possible binding to protein, this diphosphonate has a high affinity for bivalent cations, such as Ca^{++} and Mg^{++} [25]. Even though part of the circulating form of EHDP would be a mixture of the anionic species, one would not expect that the phenomenon of passive tubular secretion by nonionic diffusion with subsequent trapping would take place because of the pK values just listed. On the other hand, modification of the urinary pH within a range of 6.10 to 7.9 could alter the proportion of the various species of EHDP present in the tubular fluid. This might change the overall tubular permeability of EHDP and thereby in-

fluence its excretion. However, our findings do not show an influence of urinary pH on the renal handling of EHDP. Furthermore, in experiments not reported here, we have found no additional fall in urinary pH when the dose of NH_4Cl was increased twofold. Under these latter conditions, there was an increased fall in C_{In}, but C_{EHDP}/C_{In} was not significantly different from the data presented in Table 3.

Second, the effect of high doses of substances belonging to the two well-described renal secretory systems [20] were tested on C_{EHDP}. PAH and probenecid belong to the system secreting several organic acids [26], whereas N-methylnicotinamide (NMN) shares the system which secretes organic bases [26]. As far as their free circulating form, at pH 7.4, is considered, these compounds should be entirely dissociated into monovalent anions (cations) as their pKa (pKb) values are as follows: PAH, 3.8; probenecid, 3.4 [27]; NMN, strong base, [28]. None of these substances altered C_{EHDP} and neither did Ca-EDTA, which has been suggested to be secreted at least in the rat by a third separate system [20, 29] about which there is still some controversy [30].

Inorganic phosphate (P_i) has very recently been reported to be secreted in the rat nephron [22], but its secretory pathway has not yet been identified. P_i is dissociated at pH 7.4 mainly into its monovalent and bivalent forms, and except for the fraction which is complexed by sodium and divalent cations, P_i in the plasma is ionized [31]. Of interest is our finding that an infusion of P_i depressed C_{EHDP}, the mechanism as yet being unknown. Among several interpretations, one could speculate that diphosphonates and P_i share a common transport mechanism at the renal tubular level. This effect could also be due to a reduced glomerular ultrafiltrability of EHDP. *In vitro* experiments would support this latter possibility as the ultrafiltrable fraction of EHDP was markedly lowered in plasma of P_i-loaded rats.

The question arises whether these results have a relevance with respect to the hyperphosphatemia seen in humans treated with EHDP. Either P_i and EHDP share a common transport mechanism for secretion as discussed above or, alternatively, they may inhibit the transport of each other. However, in rats, while P_i decreases C_{EHDP}, the opposite could not be demonstrated in our conditions by either chronic subcutaneous or acute i.v. administration of variable doses of EHDP in unloaded and P_i-loaded intact animals (unpublished data). It still remains possible that the inhibition of one compound is not of the same magnitude in the two species, so that the plasma concentrations of EHDP obtained in the rat would not be sufficient to inhibit P_i transport.

Finally, the other diphosphonate, Cl_2MDP, which is also shown in the present work to be secreted, was found to affect C_{EHDP}. Therefore, one can suspect that the influence of Cl_2MDP on C_{EHDP} reflects the existence of a common renal transport pathway shared by these two diphosphonates.

In conclusion, these studies demonstrate that diphosphonates are secreted by the renal tubule. The secretory mechanism involved is as yet unknown. However, it appears to be different from those handling organic acids, organic bases and EDTA.

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