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# Disposition of 4-Methylbenzoylglycine in Rat Isolated Perfused Kidney and Effects of Hippurates on Renal Mitochondrial Metabolism

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**Abstract**

Hippurates tend to accumulate within proximal tubule cells during renal secretion. High intracellular concentrations can alter proximal tubular function or lead to tubular toxicity. In this study we examined the renal disposition of the hippurate 4-methylbenzoylglycine, a compound known for its high renal intrinsic clearance in-vivo. The effect of intracellular accumulation on mitochondrial respiration was also measured in-vitro and compared with that of the 2-methyl and 4-amino analogues.

Experiments were performed with either 2·5% pluronic or a combination of 2·2% pluronic and 2% bovine serum albumin (BSA) as oncotic agents. Within the concentration range studied ($1-200\,\mu g\,mL^{-1}$) tubular secretion seemed to be a function of the amount of unbound drug in the perfusate. Renal excretion data were best fitted by a model in which a Michaelis–Menten term was used to describe active secretion. Parameters obtained after the analysis of renal excretion data were the maximum transport velocity ($T_M = 55 \pm 2\,\mu g\,min^{-1}$) and the Michaelis–Menten constant for tubular transport ($K_T = 4{\cdot}2 \pm 0{\cdot}8\,\mu g\,mL^{-1}$). The compound accumulated extensively in kidney tissue, ratios up to 600 times the perfusate concentration were reached. Accumulation could be explained by active tubular uptake and data were analysed best by a model similar to the model used to describe renal excretion. Calculated parameters were theoretical maximum capacity ($R_M = 300 \pm 210\,\mu g\,g^{-1}$) and affinity constant for renal accumulation ($K_A = 5{\cdot}0 \pm 4{\cdot}4\,\mu g\,mL^{-1}$). The high intracellular concentrations of 4-methylbenzoylglycine had no effect on kidney function and mitochondrial oxygen consumption. The 2-methyl analogue reduced mitochondrial respiration slightly, but 4-aminobenzoylglycine (*p*-aminohippurate) caused a significant reduction.

In conclusion, this study shows that renal accumulation of a hippurate is determined by the efficiency of its tubular secretion. Whether the high intracellular concentrations affect tubular cell functioning depends on the analogue involved.

Hippurates (benzoylglycines) are known to be cleared very rapidly by the kidney by means of active secretion mediated by the organic-anion-transport system in proximal tubules. *p*-Aminohippurate has been considered as a model compound; because it undergoes extensive secretion and is only minimally bound to plasma proteins (Grantham & Chonko 1991) renal *p*-aminohippurate clearance is a suitable measure of renal tubular function (Chasis et al 1945).

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To determine the influence of ring substitution on the excretion of hippurate, Russel et al (1989a, b) studied the in-vivo renal clearance of a series of *ortho-*, *meta-* and *para-*substituted benzoylglycines by the dog. A large diversity in excretory characteristics was observed, but in general it was concluded that introduction of a substituent into the benzoylglycine ring results in a compound for which the kidney has a higher clearance capacity. Two compounds of special interest emerged from these studies—2-methylbenzoylglycine, because of its tight protein binding and exceptional clearance profile, and 4-methylbenzoylglycine, because its intrinsic clearance was highest, almost fourfold that of *p*-aminohippurate.

In a previous study we examined the renal secretion and accumulation kinetics of 2-methylbenzoylglycine in the rat isolated perfused kidney (Masereeuw et al 1996a). We observed that despite a high intrinsic clearance, active tubular secretion seemed to be a function of unbound drug in perfusate and was best described by saturable kinetics consisting of high- and a low-affinity Michaelis–Menten terms. Extensive accumulation of the drug in kidney tissue seemed to be directly related to the kinetics of active tubular uptake. This is in good agreement with previous findings that hippurates accumulate in proximal tubular cells during transepithelial secretion, as a consequence of active cellular uptake (Forster & Copenhaver 1956; Tune et al 1969; Schäli & Roch-Ramel 1981; Cox et al 1989; Masereeuw et al 1994).

Although the membrane steps for secretion have been studied widely, relatively minor attention has been paid to the intracellular disposition of hippurates. Two decades ago *p*-aminohippurate was found to interact with the anion-binding protein ligandin and it was suggested that this protein plays a role in reducing the free cytoplasmic concentration (Kirsch et al 1975). More recently it was shown that compartmentalization of organic anions within cellular organelles can also occur during secretion. The intracellular disposition of fluorescein, a fluorescent model compound of *p*-aminohippurate transport, was visualized by use of confocal microscopy (Masereeuw et al 1994, 1996b). The results of these investigations revealed that fluorescein is sequestered in mitochondria and vesicular structures of yet unidentified origin. Mitochondrial accumulation might be accompanied by a reduction in cellular energy metabolism and, therefore, high intracellular concentrations of organic anions in the proximal tubule might have harmful effects on tubule cell functioning. At present, however, it is not clear whether mitochondrial accumulation occurs with organic anions other than fluorescein during renal secretion.

In this study we examined the renal secretion and accumulation characteristics of 4-methylbenzoylglycine in the rat isolated perfused kidney. We also studied the effect of 4-methylbenzoylglycine and its 2-methyl and *p*-amino analogues on mitochondrial energy metabolism.

## Materials and Methods

Pluronic F-108 was obtained from BASF (Arnhem, The Netherlands), bovine serum albumin (BSA) was purchased from Boehringer (Mannheim, Germany) and inulin from Sigma (St Louis, MO). 4-Methylbenzoylglycine was synthesized as described elsewhere (Russel et al 1989a). Other chemicals were of analytical grade and obtained from Sigma or Merck (Darmstadt, Germany).

### *Experimental procedure*

The isolation and perfusion of the rat kidney have been described in detail elsewhere (Masereeuw et al 1996a). Studies were performed with perfusate containing either 2·5% pluronic or a combination of 2% BSA and 2·2% pluronic. For determination of glomerular filtration rate (GFR) cyanocobalamin (20 $\mu$g mL$^{-1}$) was added to the perfusion fluid and GFR was monitored on-line by use of a micro flow-through cuvette. In the presence of BSA, inulin (100 $\mu$g mL$^{-1}$) was used to determine GFR. Doses of 4-methylbenzoylglycine added to the perfused kidneys were 0·625, 1·875, 6·25, 18·75, 37·5 or 62·5 mg in 250 mL perfusion fluid.

### *Mitochondrial respiration measurements*

The effect of 4- and 2-methylbenzoylglycine and *p*-aminohippurate on cellular energy metabolism was assessed by measuring mitochondrial respiration in the absence of adenosine diphosphate (ADP) (state 2), in the presence of ADP (state 3), after consumption of ADP (state 4), and after the addition of dinitrophenol. To this end, rat kidney cortex mitochondria were isolated as described elsewhere (Masereeuw et al 1996b). All steps were performed at 4°C. Briefly, kidneys were isolated after perfusion with ice-cold solution containing 140 mM NaCl and 10 mM KCl. The capsula was removed, the medulla was dissected and the cortex was transferred to a Potter–Elvehjem homogenizer with Teflon pestle (clearance 0·5 mm) in three times the tissue weight of homogenization buffer (300 mM mannitol, 10 mM HEPES, 1 mM EGTA, 1 mg mL$^{-1}$ BSA, pH 7·4). Tissue was homogenized gently six times by hand, and the resulting suspension was centrifuged for 10 min at 500 *g*. The supernatant was collected and centrifuged for 7 min at 11 000 *g*. The pellet was washed with homogenization buffer and again centrifuged (7 min, 11 000 *g*). The final pellet was diluted to a concentration of 5 mg mL$^{-1}$ protein in respiration medium (210 mM mannitol, 10 mM KCl, 10 mM $KH_2PO_4$, 0·5 mM EGTA, 60 mM Tris–HCl, pH 7·4). Oxygen consumption was measured at 30°C with a Clarke-type platinum electrode, using 1 mg mitochondrial protein in 2·0 mL respiration medium. Succinate (10 mM) was used as the metabolic substrate, and rotenone (1 $\mu$M) was added to block electron transport proximal to succinate entry into the respiratory chain. ADP-stimulated respiration (state 3) was measured in the presence of 0·3 mM ADP. Dinitrophenol (44 $\mu$M) was used as uncoupling agent.

Respiratory rates were calculated and expressed as nanogram atoms of oxygen $min^{-1}$ (mg mitochondrial protein)$^{-1}$ (ng atom O $min^{-1}$ (mg protein)$^{-1}$).

*Analysis*

Urine and perfusate samples were analysed for glucose and different electrolytes as described elsewhere (Cox et al 1990). In the presence of 2% BSA, perfusate and urine samples were also analysed for inulin and protein content. The Bio-Rad Protein Assay from Bio-Rad (Munich, Germany) was used for the determination of protein. Inulin was determined according to a previously published method (Heyrovski 1956). The concentration of 4-methylbenzoylglycine in perfusate, urine and kidney samples was determined by reversed-phase high-performance liquid chromatography (HPLC), essentially based on a method described elsewhere for 2-methylbenzoylglycine (Masereeuw et al 1996a). The only modification was the use of a UV-absorbance wavelength of 238 nm.

*Protein binding*

Protein binding was determined by ultrafiltration as described elsewhere (Russel et al 1987). The ultrafiltrates were treated and analysed in the same way as the urine samples. Perfusate protein binding, assuming one class of binding site, was calculated according to the equations:

$$C = Cu + P \cdot Cu/(K_d + Cu) \quad (1)$$

$$Cu = fu \cdot C \quad (2)$$

in which C is the total perfusate concentration ($\mu g\, mL^{-1}$), Cu is the concentration of unbound drug ($\mu g\, mL^{-1}$), fu is the fraction of unbound drug in perfusate, P is the total concentration of protein binding sites ($\mu g\, mL^{-1}$), and $K_d$ is the dissociation constant of the drug–protein complex ($\mu g\, mL^{-1}$).

*Renal excretion model*

The renal clearance of 4-methylbenzoylglycine by the rat isolated perfused kidney could be described by a model comprising glomerular filtration and tubular secretion according to Michaelis–Menten kinetics. Assuming that renal excretion of 4-methylbenzoylglycine is dependent on unbound perfusate concentrations, renal clearance, $CL_R$, can be expressed as:

$$CL_R = (Q_{GF} \cdot fu \times [T_M \cdot fu/(K_T + Cu)] \times (1 - F) \quad (3)$$

The renal excretion rate ($R_R$) is:

$$R_R = CL_R \cdot C \quad (4)$$

where $CL_R$ is the renal clearance (mL $min^{-1}$), $R_R$ is the renal excretion rate ($\mu g\, min^{-1}$), $Q_{GF}$ is the glomerular filtration rate (mL $min^{-1}$), C is the total drug concentration in perfusate ($\mu g\, mL^{-1}$), Cu is the concentration of unbound drug in perfusate ($\mu g\, mL^{-1}$), fu is the fraction of unbound drug, $T_M$ is the maximum transport velocity ($\mu g\, min^{-1}$), $K_T$ is the Michaelis–Menten constant for tubular transport ($\mu g\, mL^{-1}$), and F is the fraction of excreted drug reabsorbed.

*Renal accumulation*

The concentration of 4-methylbenzoylglycine in the kidney ($C_T$) was expressed as the amount of drug/unit weight of tissue. The concentration in kidney tissue divided by the concentration in perfusate at the end of the experiment (Cu,z), resulted in an accumulation ratio (kidney/perfusate ratio). If accumulation in kidney tissue is considered to be a result of both active and passive transport processes, the renal accumulation ratio can be described by:

$$C_T/Cu, z = R_M/(K_A + Cu, z) + a \quad (5)$$

where $R_M$ is the theoretical maximum capacity ($\mu g\, g^{-1}$), $K_A$ is the affinity constant for renal accumulation ($\mu g\, mL^{-1}$), and a is the ratio $C_T$/Cu,z as a result of passive transport.

*Data analysis*

Renal excretion rate and accumulation data were analysed according to equations 4 and 5 by means of the non-linear least-square regression program PCNonlin (Metzler & Weiner 1986). The goodness of fit was evaluated as the deviation between the observed and model predicted values as $R^2 = 1 - \sum(Dev)^2 / \sum(Obs)^2$, where $\sum(Obs)^2$ is the sum of squared observations and $\sum(Dev)^2$ the sum of squared deviations. All data are expressed as means ± standard deviation (s.d.). The statistical significance of differences between means was determined with Student's *t*-test. In respiration measurements, the multiple means were compared by use of one-way analysis of variance followed by the least significant difference post-hoc test. The level of significance was set to $P < 0.05$.

## Results

*Effects on kidney function*

Renal functional parameters from 12 control experiments in the absence and presence of BSA, and after administration of the highest dose of 4-methylbenzoylglycine, are listed in Table 1. In the presence of 2% BSA GFR was lower, owing to enhanced oncotic pressure within the glomerulus

and peritubular capillaries. As a result of a lower perfusate viscosity, a higher perfusate flow was necessary to maintain normal pressure. The fractional excretion (%) of most electrolytes and glucose was reduced in the presence of BSA. No negative effects on kidney function were observed up to the highest dose of 62·5 mg.

*Protein binding*

Protein binding in the perfusate was determined for each dose of 4-methylbenzoylglycine administered and found to be concentration-dependent. The unbound fraction varied between 0·1 and 0·7 over the concentration range 0·5–150 $\mu$g mL$^{-1}$. Protein binding data from all experiments were pooled and analysed according to equation 2. The parameters obtained were a dissociation constant, $K_d$, of $9 \pm 2\,\mu$g mL$^{-1}$ and a total concentration of BSA binding sites, P, of $35 \pm 4\,\mu$g mL$^{-1}$ (n = 18; $R^2 = 0{\cdot}984$).

*Renal excretion and accumulation*

4-Methylbenzoylglycine was slowly eliminated from perfusate. Figure 1 shows the mean concentration of unbound perfusate and corresponding excretion rate data as functions of time under both experimental conditions. All doses gave log–linear concentration–time curves. The urinary excretion rate increased rapidly after addition of 4-methylbenzoylglycine; this was followed by a gradual decrease. The presence of protein in the perfusate resulted in lower renal excretion rates for all doses. Because 4-methylbenzoylglycine is known to undergo deconjugation in the dog (Russel et al 1989a) plasma and urine samples were examined for the presence of 4-methylbenzoate, but this compound was not detected.

Renal handling data for 4-methylbenzoylglycine are presented in Table 2. At low perfusate concentrations the renal clearance was many times higher than the clearance by glomerular filtration

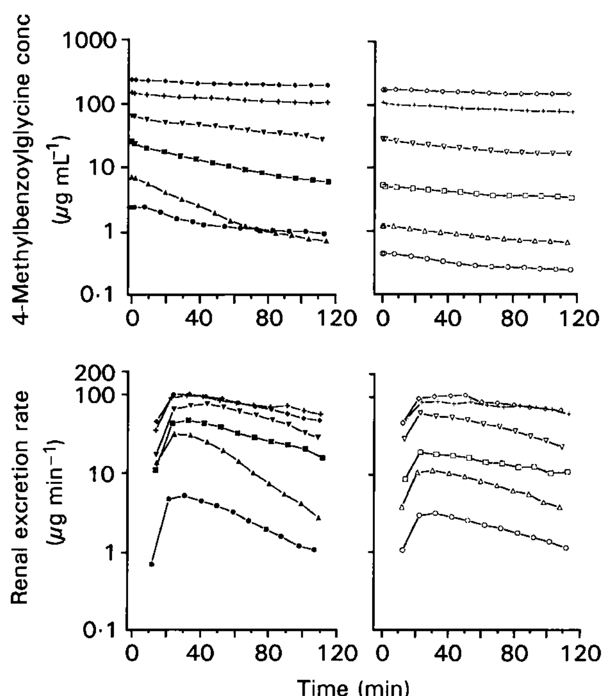


Figure 1. Concentration of unbound 4-methylbenzoylglycine in perfusate (upper) and urinary excretion rate (lower) as a function of time in the rat isolated perfused kidney. Closed and open symbols represent, respectively, experiments without and with BSA in the perfusate. ●, ○, 0·625; ▲, △, 1·875; ■, □, 6·25; ▼, ▽, 18·75; +, +, 37·5; ◆, ◇, 62·5 mg 4-methylbenzoylglycine. All data points are means of results from four experiments. For the sake of clarity, standard deviations were omitted from this figure; they varied between 20 and 51% for low perfusate concentrations (0–25 $\mu$g mL$^{-1}$), between 1 and 30% for high concentrations (25–250 $\mu$g mL$^{-1}$) and between 6 and 39% for renal excretion rate.

Table 1. Functional parameters of the rat isolated perfused kidney in control experiments and after administration of 62·5 mg 4-methylbenzoylglycine.

| Parameter | 2·5% Pluronic | | 2·2% Pluronic + 2% bovine serum albumin | |
|---|---|---|---|---|
| | Control (n = 12) | 4-Methylbenzoylglycine (n = 4) | Control (n = 12) | 4-Methylbenzoylglycine (n = 4) |
| Fractional excretion sodium (%) | 2·9 ± 0·6 | 2·8 ± 1·0 | 3·1 ± 1·7 | 2·9 ± 0·8 |
| Fractional excretion potassium (%) | 38 ± 14 | 40 ± 14 | 14 ± 6† | 22 ± 7 |
| Fractional excretion glucose (%) | 7·3 ± 2·1 | 8·9 ± 1·8 | 4·1 ± 2·0† | 6·0 ± 0·6 |
| Fractional excretion magnesium (%) | 42 ± 10 | 22 ± 4* | 17 ± 12† | 18 ± 5 |
| Fractional excretion calcium (%) | 3·5 ± 1·3 | 4·0 ± 1·4 | 2·4 ± 1·3 | 2·6 ± 0·6 |
| Water re-absorption (%) | 94 ± 1 | 93 ± 1 | 93 ± 1 | 93 ± 1 |
| Urine flow ($\mu$L min$^{-1}$) | 18 ± 3 | 19 ± 3 | 16 ± 3 | 15 ± 1 |
| Glomerular filtration rate ($\mu$L min$^{-1}$) | 277 ± 35 | 284 ± 15 | 232 ± 53† | 234 ± 44 |
| Urinary pH | 5·8 ± 0·3 | 6·0 ± 0·1 | 6·2 ± 0·2† | 6·3 ± 0·03 |
| Perfusate flow (mL min$^{-1}$) | 15 ± 2 | 14 ± 2 | 23 ± 2† | 23 ± 3 |
| Perfusate pressure (mm Hg) | 89 ± 8 | 80 ± 3 | 93 ± 8 | 97 ± 4 |

Values are means ± s.d. over the 30–120 min period. *$P < 0{\cdot}05$, significantly different from control result; †$P < 0{\cdot}05$, significantly different from result from corresponding experiment without bovine serum albumin.

(GF) corrected for the unbound fraction ($CL_R > GF$), indicating active tubular secretion. The decrease in $CL_R/GF$ at higher perfusate concentrations is an indication of saturation of tubular secretion. The $CL_R/GF$ values in the presence of BSA exceeded those obtained from the corresponding experiments with 2·5% pluronic, supporting the view that the renal excretion of 4-methylbenzoylglycine is dependent on unbound drug concentrations. Therefore, clearance data from the experiments with BSA in perfusion fluid were pooled with those from the experiments in the absence of BSA and analysed simultaneously. The tubular titration curve in Figure 2 represents the relationship between renal excretion rate and unbound perfusate concentration at the midpoint of a urine collection interval. The plot gives a clear impression of the processes involved in the clearance of renal 4-methylbenzoylglycine. The compound undergoes net tubular secretion which becomes saturated at higher perfusate concentrations. At these concentrations the titration curve runs parallel to the glomerular filtration (GF) line, indicating that passive re-absorption of 4-methylbenzoylglycine was negligible (F = 0 in equation 3). The line through the data points was obtained after analysing renal excretion data over the period 30–90 min, according to equation 4. The kinetic parameters determined after fitting equation 4 to the data were a maximum transport velocity, $T_M$, of $55 \pm 2\,\mu g\,min^{-1}$ and a Michaelis–Menten constant for tubular transport, $K_T$, of $4·2 \pm 0·8\,\mu g\,mL^{-1}$.

At the end of each experiment the concentration of drug in kidney tissue was determined (Table 2). Concentrations up to 600 times the perfusate concentration were observed at low perfusate concentrations (Figure 3). Values for individual kidneys are presented because of the high variation at low concentrations of the medium. After fitting equation 5 to the accumulation data, the kinetic parameters for renal accumulation were a theoretical maximum capacity, $R_M$, of $300 \pm 210\,\mu g\,g^{-1}$, an affinity constant for renal accumulation, $K_A$, of $5·0 \pm 4·4\,\mu g\,mL^{-1}$ and an accumulation ratio as a result of passive transport, a, of $4·1 \pm 3·1$.

*Mitochondrial respiration*

The effect of 4-methylbenzoylglycine on mitochondrial oxygen consumption was determined and compared with the effects of two other hippurates, 2-methylbenzoylglycine and *p*-aminohippurate, known to accumulate in proximal tubular cells. The respiratory control ratio (RCR) in Table 3 indicates that our mitochondrial preparation was of good quality and tightly coupled. A concentration of

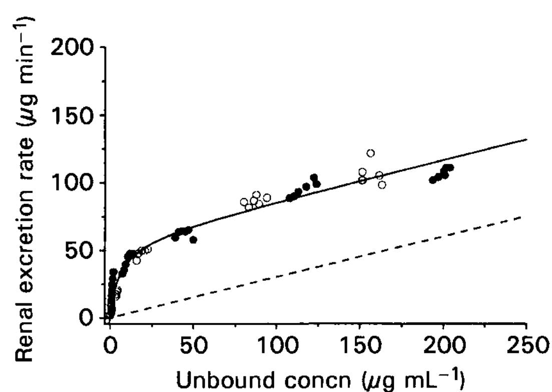


Figure 2. Tubular titration curve for 4-methylbenzoylglycine renal excretion rate and rate of filtration as a function of unbound concentration in the perfusate of the rat isolated perfused kidney. The solid line represents the fit according to equation 4, the dashed line corresponds to clearance by glomerular filtration (GF) only. Data obtained between 30 and 90 min under both sets of experimental conditions were pooled and analysed simultaneously. ●, without BSA; ○, with BSA. All data points are mean values from four experiments.

Table 2. Renal handling of 4-methylbenzoylglycine in the rat isolated perfused kidney.

| Dose (μg) | Bovine serum albumin | Perfusate concn (μg mL⁻¹) | Fraction unbound | Excretion rate (μg min⁻¹) | $CL_R/GF$[a] | Amount in kidney (μg g⁻¹) |
|---|---|---|---|---|---|---|
| 625 | – | 1·2±0·2 | – | 5·8±1·1 | 11±1 | 20±7 |
| | + | 1·7±0·2 | 0·17 | 2·6±0·4 | 25±3 | 90±38 |
| 1875 | – | 1·6±0·8 | – | 24±8 | 26±3 | 46±13 |
| | + | 4·5±0·5 | 0·18 | 10±2 | 40±2 | 72±20 |
| 6250 | – | 11±3 | – | 41±6 | 9·2±0·9 | 235±102 |
| | + | 17±1 | 0·23 | 19±2 | 13·6±0·4 | 112±40 |
| 18750 | – | 45±5 | – | 59±8 | 4·0±0·4 | 487±76 |
| | + | 46±4 | 0·42 | 49±3 | 7·9±0·7 | 391±55 |
| 37500 | – | 117±7 | – | 94±7 | 2·3±0·1 | 793±118 |
| | + | 123±7 | 0·72 | 87±3 | 3·1±0·2 | 540±51 |
| 62500 | – | 201±4 | – | 106±6 | 1·29±0·03 | 1027±149 |
| | + | 193±6 | 0·81 | 104±10 | 2·4±0·2 | 882±90 |

Values are means ± s.d. over the 30–90 min period for both sets of experimental conditions (n = 4). [a]Renal clearance of 4-methylbenzoylglycine corrected for glomerular filtration rate and fraction unbound in perfusate.

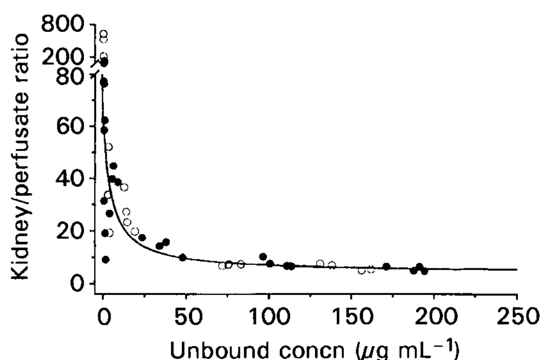


Figure 3. Accumulation of 4-methylbenzoylglycine in rat kidney tissue. The kidney/perfusate ratio is plotted against the unbound perfusate concentration after fitting the data according to equation 5. ●, without BSA; ○, with BSA. All data points are for individual kidneys; n = 24 for both sets of experimental conditions.

100 μM 4-methylbenzoylglycine was used, which is similar to a free cytoplasmic concentration of 518 μg $mL^{-1}$ and in agreement with kidney tissue concentrations determined at an intermediate dose (Table 2). At this concentration the drug did not alter succinate-stimulated respiration, either at basal respiration or after addition of ADP. No effect on uncoupled respiratory rate was observed (dinitrophenol). Using the same concentration of 2-methylbenzoylglycine a slight but not significant decrease in ADP-stimulated respiration (state 3) was observed, and, as a consequence, RCR decreased. *p*-Aminohippurate elicited a significant reduction in basal respiration, state 3 respiration and RCR at 100 μM, suggesting that the availability of succinate for oxidation was reduced.

## Discussion

The results of our study show that excretion of 4-methylbenzoylglycine by the perfused rat kidney is determined by glomerular filtration and active, saturable secretion. Despite the large accumulation in kidney tissue the compound had no effect on renal function. Plasma protein binding of 4-methylbenzoylglycine was concentration-dependent, which affected its renal clearance. The maximum clearance capacity of the secretory system, as described by the intrinsic clearance ($CL_{int} = T_M/K_T$), was 13 mL $min^{-1}$, which is higher than previously determined values of 10 mL $min^{-1}$ for 2-methylbenzoylglycine (Masereeuw et al 1996a) and 4 mL $min^{-1}$ for 2-hydroxybenzoylglycine (salicyluric acid; Cox et al 1989). These findings are in accordance with in-vivo studies in the dog (Russel et al 1989a, b).

The Michaelis–Menten constant for secretion in the rat isolated perfused kidney, 4 μg $mL^{-1}$, is in agreement with a value of 8 μg $mL^{-1}$ reported for the dog (Russel et al 1989a). According to Cox et al (1991) the maximum transport velocities, $T_M$, of hippurates in rat and dog kidney are comparable assuming that $T_M$ is a linear function of tubular cell volume. For the rat kidney the ratio of $T_M$ to cell volume (i.e. 340 μL) is 162, and for the dog kidney this ratio is 267 (cell volume is approximately 30 mL) (Russel et al 1989a). Apparently, the pharmacokinetic parameters found for the rat isolated perfused kidney are similar to values found for the dog.

In contrast with earlier findings with the dog (Russel et al 1989a), however, tubular secretion of 4-methylbenzoylglycine was a function of the concentration of unbound drug in the perfusion fluid. As a result of the high perfusion flow in our perfused kidney preparations (14 mL $min^{-1}$ in the absence of BSA and 23 mL $min^{-1}$ in the presence of 2% BSA) the renal extraction ratio, calculated as $CL_{int}$, divided by the sum of $CL_{int}$ and perfusate flow, is relatively low, notwithstanding the efficient

Table 3. Effect of substituted hippurates on succinate-stimulated respiration in rat kidney cortex mitochondria.

| Compound | Respiratory rate (ng atom O $min^{-1}$ (mg protein)$^{-1}$) | | | | |
|---|---|---|---|---|---|
| | State 2 | State 3 | State 4 | Dinitrophenol[a] | Respiratory control ratio[b] |
| Control | 29 ± 6 | 93 ± 22 | 28 ± 7 | 81 ± 20 | 3·4 ± 0·5 |
| 4-Methylbenzoylglycine | 32 ± 3 | 94 ± 13 | 30 ± 7 | 76 ± 30 | 3·3 ± 0·7 |
| 2-Methylbenzoylglycine | 31 ± 4 | 74 ± 23 | 28 ± 8 | 71 ± 28 | 3·0 ± 0·5 |
| *p*-Aminohippurate | 19 ± 5* | 68 ± 8* | 27 ± 8 | 75 ± 26 | 2·6 ± 0·4* |

Values are means ± s.d. Succinate-dependent respiration was measured in mitochondria pre-incubated for 3 min in respiration buffer in the presence of the compound tested (100 μM). Oxygen consumption was measured before addition of adenosine diphosphate (state 2), after addition of 0·3 mM adenosine diphosphate (state 3) and after consumption of adenosine diphosphate (state 4). [a]Uncoupled respiratory rate was measured in the presence of 44 μM dinitrophenol. [b]Respiratory control ratio is the ratio of state 3 respiration to state 4 respiration. *$P < 0·05$, significantly different from control result (one-way analysis of variance followed by the least significant differences post-hoc test).

secretion of 4-methylbenzoylglycine. Depending on the experimental conditions the renal extraction ratio varied between 0·4 and 0·5, indicating that in the rat isolated perfused kidney 4-methylbenzoylglycine is handled as an intermediate clearance drug.

The Michaelis–Menten constant for renal accumulation is in good agreement with the value for renal excretion, suggesting that the same active mechanism is involved. It is probable that the basolateral organic anion system in proximal tubules mediates cellular uptake of 4-methylbenzoylglycine (Grantham & Chonko 1991; Pritchard & Miller 1996). The accumulation we found for 4-methylbenzoylglycine of up to 600 times the concentration of the medium is high compared with a maximum accumulation ratio of 175 determined for 2-methylbenzoylglycine (Masereeuw et al 1996a), and 20 for 2-hydroxybenzoylglycine (Cox et al 1989). These results suggest that anionic drugs with a high intrinsic clearance might reach high intracellular concentrations during renal excretion.

The high intracellular concentrations of 4-methylbenzoylglycine were without harmful effects on kidney functional parameters. Proximal tubule cell function remained stable throughout the experimental period up to the highest dose administered to the perfused kidney, as can be assessed from the fractional excretion of glucose. We examined the effect on cellular energy metabolism, because previous studies with the organic anion fluorescein (a diagnostic agent transported by the *p*-aminohippurate carrier) have shown that mitochondrial accumulation occurs during renal tubular secretion, which might be accompanied by a reduction in oxidative metabolism (Masereeuw et al 1994, 1996b). In the current study 4-methylbenzoylglycine did not affect mitochondrial oxygen consumption, which is a good indication that the compound does not interfere with mitochondrial metabolism. 2-Methylbenzoylglycine affected mitochondrial oxygen consumption slightly, but not significantly, which is in agreement with its moderate effect on kidney function (Masereeuw et al 1996a). *p*-Aminohippurate, however, significantly inhibited both state 2 and state 3 of succinate-stimulated respiration. Whether *p*-aminohippurate accumulates within mitochondria as a result of specific uptake by one of the metabolite anion carriers remains to be elucidated. A consequence of reduced mitochondrial oxygen consumption is diminished ATP production, and long-term exposure to the drug might result in the development of tubular toxicity. The reason tubular cell injury has never been reported for *p*-aminohippurate is probably because of the low concentrations used clinically compared with the high concentrations used in this study.

In conclusion, 4-methylbenzoylglycine is handled as an intermediate clearance drug in the rat isolated perfused kidney, and, in contrast with in-vivo results, renal clearance seemed to be a function of the concentration of unbound drug in the perfusion medium. The compound accumulated extensively in kidney tissue as a result of active tubular uptake, mediated by the secretory mechanism. The results suggest that anionic drugs with a high intrinsic clearance reach high intracellular concentrations. No effect on kidney function and mitochondrial oxygen consumption was observed for intracellularly accumulated 4-methylbenzoylglycine, but its structural analogue *p*-aminohippurate significantly affected mitochondrial metabolism.

*Acknowledgement*

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