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Ms. Susan Wayland  
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Office of Prevention, Pesticides and Toxic Substances TS-7101  
Environmental Protection Agency  
401 M Street, SW,  
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Washington, DC 20460

**Contains No CBI**

Dear Ms. Wayland:

The Chemical Manufacturers Association makes available to the public and appropriate government agencies final reports of environmental, health, and safety research that it manages. In keeping with this policy, the following final report that the CMA Ethylene Glycol Ethers Panel recently conducted is enclosed:

"Species-, Gender-, and Age-Related Differences in the Pharmacokinetics of 2-Butoxyethanol and 2-Butoxyacetic acid. II. *In Vitro* Renal Active Transport of 2-Butoxyacetic Acid"

The report does not include confidential information.

If you have any questions, please call Dr. Susan Lewis of my staff at 703-741-5635.



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Enclosure

Sincerely yours,

Courtney M. Price/HCS

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Battelle Project No. 23529

CMA Agreement No. EGE-74.0-DOSE-BAT.NW

October 25, 1999

**“Species-, Gender-, and Age-Related Differences in the Pharmacokinetics of 2-**

**Butoxyethanol and 2-Butoxyacetic acid.**

**II. *In Vitro* Renal Active Transport of 2-Butoxyacetic Acid”**

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### ABSTRACT

2-Butoxyacetic acid (BAA), a metabolite of 2-butoxyethanol (BE), is responsible for the observed hemolytic toxicity of BE in laboratory animals. BE-induced hemolysis occurs in a species-, age-, and gender-dependent manner, which can be explained, in part, by differences in red blood cell susceptibility to lysis. Additional differences in rates of formation, distribution, and elimination of BAA may also significantly contribute to its hemolytic potential. Studies have suggested that BAA is actively secreted in the kidney and may account for some of the differences observed in toxicity. The objective of the present study was to characterize the age-, species-, and gender-dependent influences on the renal elimination of BAA. *In vitro* estimates of renal acid secretion of BAA were determined using precision cut renal slices from young and old, male and female, rats and mice. Time-course studies were conducted in which renal slices were incubated with various concentrations of <sup>14</sup>C-labeled BAA alone or in combination with probenecid in a dynamic culture system. The data indicate marked differences between rats and mice with respect to the active transport of BAA, as well as possible age- and gender-dependent differences in rats. Renal active transport of BAA did not differ between young and old male rats, but did differ among female rats. Gender-dependent differences were observed in both old and young rats, where females have a lower affinity, but higher capacity pathway compared to male rats of similar age. In contrast, mice have only a marginal ability to actively transport BAA in the kidney. These data indicate that species-, age- and gender-differences in hemolysis resulting from exposure to BAA could be attributable, in part, to differences in the active secretion of BAA.

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## INTRODUCTION

Animal exposure studies indicate several toxicological findings, including hemolysis, decreased body weight gains, liver and kidney toxicity (secondary to hemolysis), and skin irritation (Tyler, 1984). The acute hemolytic anemia has been reported to occur in a dose-, species-, age-, and gender-dependent manner (Smyth *et al.*, 1941; Carpenter *et al.*, 1956; Tyler, 1984; Ghanayem *et al.*, 1987a, 1990; Ghanayem, 1989; Ghanayem and Sullivan, 1993; Udden, 1994; Udden and Patton, 1994). In general, studies suggest that older animals are more sensitive than younger animals (Tyler 1984, Ghanayem *et al.* 1987a, 1990), females are more sensitive than males (Udden and Patton 1994), and rodents are significantly more sensitive than humans (Carpenter *et al.* 1956, Udden and Patton 1994).

The primary BE metabolite, butoxyacetic acid (BAA), has been shown to be responsible for the hemolysis of red blood cells (Ghanayem, 1989; Ghanayem and Sullivan, 1993; Udden and Patton, 1994; Udden, 1994). Factors which may contribute to the species differences in toxicity include both pharmacodynamics (sensitivity of red blood cells to the internal dose of BAA) and pharmacokinetics (absorption, distribution, metabolism and elimination). For example, Grant *et al.* (1999) observed species-, gender-, and age-dependent differences in BAA formation from BE via the alcohol/aldehyde dehydrogenase pathway similar to the age-related differences reported in male rats by Ghanayem *et al.* (1987a, 1990).

Pharmacokinetic differences between species have been the focus of efforts to improve human health risk assessments for BE and its hemolytic metabolite BAA. To this end, a PBPK model was developed to describe the disposition of BE and BAA in male rats and humans (Corley *et al.*, 1994). Sensitivity analyses of this model demonstrated that parameters describing

the formation of BAA, its distribution (protein binding) and elimination (renal active transport), were critical parameters in accurate predictions of internal dose estimates of BAA (Lee *et al.*, 1995). Furthermore, a chronic bioassay in F344 rats and B6C3F1 mice in progress at the National Toxicology Program includes groups of animals to evaluate the kinetics of BE over the course of the bioassay. Therefore, it is important to expand the current PBPK model to include both genders of mice and rats, as well as the potential impacts of aging.

The focus of the present study was to determine the effect of age, gender, and species on the renal active transport of BAA, using precision-cut renal slices. This methodology offers the advantage of maintaining tissue architecture, and provides for easier cross-species comparison than alternative *in vitro* systems (Bach *et al.*, 1996). In a companion paper (Grant *et al.*, 1999) the effect of age, gender, and species on the formation of BAA is described.

## MATERIALS AND METHODS

**Animals.** Young adult male and female F-344 rats (8-10 weeks of age) and young adult male and female B6C3F1 mice (4-5 weeks of age) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Old male (21 months of age), and female (18 months of age) F-344 rats, and old male B6C3F1 mice (18 months of age) were obtained through the National Institute of Aging (Bethesda, MD). Aged female mice were not available. Animals were maintained for a minimum of 5 days in 12h light:12h dark cycle in a temperature controlled animal facility and allowed food and water *ad libitum* until use.

**Chemicals.** Technical grade 2-butoxyacetic acid was donated by Shell Development Corporation (Houston, TX). Radiolabeled 2-butoxy[1-<sup>14</sup>C]acetic acid was purchased from Amersham Life Science (Buckinghamshire, England) with a radiochemical purity of 98.6% and specific activity of 16.9 mCi/mmol. All other chemicals were reagent grade and purchased from standard vendors.

**Renal slice preparation.** Animals were sacrificed by exsanguination under CO<sub>2</sub> anesthesia. Renal slices were prepared as described previously (Pritchard 1976, 1990) with the following modifications. Following excision, kidneys were stored in ice-cold, oxygenated Krebs-Henseleit/Hepes (KHH) buffer (pH=7.45) and slices (200 ± 25 µm in thickness) were prepared within 10 min of removal using a Krumdieck tissue slicer. Slices containing only the cortical section of the kidneys were discarded and the remaining slices were placed on mesh screen half cylinders, which were fitted into teflon or titanium inserts.

**Renal slice incubation.** Incubations were carried out using the dynamic organ culture method described by Smith *et al.* (1985) with the following modifications. Slices were incubated in vials containing 0.05, 0.25, 0.50 1.0 mM <sup>14</sup>C-labeled BAA in KHH buffer (95%/5% O<sub>2</sub>/CO<sub>2</sub>) with or without probenecid. Pilot studies demonstrated that at least three times more probenecid than BAA resulted in maximum inhibition of active transport, therefore a ratio of at least 3-fold (up to 10-fold) was used throughout the renal studies. Slices were removed from the buffer following 5, 15, 30, or 45 min of incubation, blotted dry on filter paper, weighed, and solubilized in 1 ml of 1M NaOH overnight, then counted in a liquid scintillation counter with Optifluor scintillation cocktail (Packard, Meriden, CT).

**Determination of slice viability and BAA toxicity.** To assess viability of control and treated liver slices, intracellular ATP and  $K^+$  content were measured in the supernatant of slices homogenized in 10% (w/v) trichloroacetic acid and centrifuged at 10,000 x g. ATP content was measured using the luciferin-luciferase assay (Kricka, 1988) as described by Hoivik *et al.* (1996) with a Turner Designs TD-20/20 Luminometer.  $K^+$  content was subsequently measured using a flame photometer (Model PFP7, Jenway, Essex, England) per the manufacturer's instructions. ATP and  $K^+$  data were compared for treatment-related differences using a one-way analysis of variance. Differences were significant if  $p \leq 0.01$ .

**Kinetic analysis.** The uptake of BAA by the kidney slice was analyzed using a two compartment model (Figure 1) similar to one used to describe the *in vitro* protein binding of methyl *t*-butyl ether in kidney homogenates (Poet and Borghoff, 1997). In this model, the transfer of free BAA between the media and the slice ( $k_1$  and  $PK$ ) and the active transport of BAA within the slice ( $K_m$  and  $V_{max}$ ) were described using the following series of equations:

$$\frac{dSLICE}{dt} = k_1 \times BAA_M - k_1 \times \frac{BAA_F}{PK} \quad (1)$$

$$SLICE = INTEG\left(\frac{dSLICE}{dt}, 0.0\right) \quad (2)$$

$$BAA_F = SLICE - BAA_E \quad (3)$$

$$\frac{dBAA_E}{dt} = V_{max} \times \frac{BAA_F}{(K_m + BAA_F)} \quad (4)$$

$$BAA_E = INTEG\left(\frac{dBAA_E}{dt}, 0.0\right) \quad (5)$$

Where  $k_1$  is the first-order transfer coefficient between media and slice ( $\text{min}^{-1}$ ), PK is the kidney:media partition coefficient,  $K_m$  (ng/mg slice) and  $V_{\text{max}}$  (ng/min/mg slice) are the Michaelis-Menten constants for renal active transport, SLICE is the total concentration of BAA in the slice (ng/mg slice),  $\text{BAA}_M$  is the concentration of BAA in the media (ng/mg media),  $\text{BAA}_F$  is the concentration of free BAA in the slice (ng/mg slice) and  $\text{BAA}_E$  (ng/mg slice) is the concentration of BAA actively transported by the slice (ng/mg slice).

To determine the  $K_m$  and  $V_{\text{max}}$  for renal active transport, the parameters driving the non-specific uptake of BAA ( $k_1$  and PK) in the two-compartment model were determined by fitting the model to the concentration-time course data for the total uptake of BAA (SLICE) in the presence of the renal active transport inhibitor, probenecid (setting  $V_{\text{max}} = 0$ ). Once the non-specific uptake parameters were determined for each set of kidney slices,  $K_m$  and  $V_{\text{max}}$  for renal active transport were determined by fitting the model to the concentration-time course data for the total uptake of BAA without probenecid. All parameter estimates were determined using the maximum likelihood estimation method of SimuSolv® (The Dow Chemical Company).

## RESULTS

### *Renal slice viability*

Control and treated slices for all experiments maintained their viability throughout the incubation period, as reflected by their  $\text{K}^+$  and ATP content. Even after 45 min of incubation

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intracellular levels of ATP and  $K^+$  levels in all slices did not differ from their respective controls (data not shown).

#### *Active secretion of BAA in renal slices from rats*

The active secretion of BAA via the renal organic anion transport system was determined *in vitro* using kidney slices prepared from young and old, male and female rats following incubation with BAA alone (active and non-specific uptake) or in combination with probenecid (non-specific uptake). There was a marked, consistent increase in the accumulation of  $^{14}C$  in all slices incubated with BAA alone compared to those co-incubated with probenecid. The difference reflects active uptake and suggests that BAA is actively secreted in the rat (Fig 2A).

Non-specific uptake of BAA from the media to the kidney slices from young male rats was analyzed using the two-compartment model (Fig 3). Single estimates of  $k_1$  and PK were determined for the model that best described all of the data from each dose level (Table 1). Similar simulations were conducted for young and old female and old male rats (simulations not shown) and  $k_1$  and PK estimates are given in Table 1.

Parameters for active transport,  $K_m$  (affinity) and  $V_{max}$  (capacity), were determined using the two-compartment model by setting  $k_1$  and PK equal to the estimates determined previously, and optimizing uptake of BAA data in slices incubated with BAA alone (Fig 4). Young and old female rats have a higher  $K_m$  and  $V_{max}$  for active transport of BAA compared to male rats of similar age (Table 1). In addition, while there was no age-dependent difference in active transport in male rats, old female rats had a lower  $K_m$  and  $V_{max}$  than did young female rats (Table 1).

*Active secretion of BAA in renal slices from mice*

Unlike rats, only a marginal time-dependent difference between slices incubated with BAA alone and slices incubated with BAA + probenecid was observed (Fig 2). The magnitude of this difference in mice indicated that active transport of BAA is much lower than observed in rats. To verify these results, additional studies, using BAA concentrations similar to those used in the probenecid experiments, were conducted at 0°C to determine if renal transport in mice occurred via an active process not inhibited by probenecid. BAA uptake in slices incubated at 0°C did not differ from uptake in slices using probenecid as the inhibitor at 37°C (data not shown) indicating that probenecid effectively inhibited renal active transport in mouse renal slices.

Estimates of non-specific uptake of BAA from the media to the kidney slice ( $k_1$  and PK) were determined as described previously using the two-compartment model and are given in Table 1 (simulations not shown). Estimates of  $K_m$  (affinity) and  $V_{max}$  (capacity) to describe the active secretion of BAA in renal slices were also determined as described for the rat (Fig 5, Table 1). There appear to be no age- or gender-related differences in  $K_m$  and  $V_{max}$  estimates of active transport of BAA within the groups of mice. However, all  $K_m$  and  $V_{max}$  estimates for mice are substantially lower than those estimated for rats corresponding to the overall marginal differences in uptake of BAA with and without probenecid.

**DISCUSSION**

Previous studies have clearly demonstrated that renal active transport plays an important role in the elimination of BAA in male F344 rats (Ghanayem *et al.*, 1990). In addition, sensitivity analyses of a PBPK model for BE and BAA in rats and humans indicated the

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importance to having reliable estimates of the rates of active transport in describing the internal dosimetry of BAA following BE exposures (Corley *et al.*, 1994; Lee *et al.*, 1995). Therefore, to expand the current PBPK model to incorporate potential age-related changes, female rats, and both genders of mice, renal active transport of BAA was investigated using a precision-cut kidney slice system coupled with a two-compartment kinetic analysis. Concentrations used in this *in vitro* study (0.05 - 1.0 mM BAA) bracketed the expected range of metabolite concentrations that could be achieved during repeated-exposure toxicity studies with rats. The highest concentration (1.0 mM) is clearly toxic to the red blood cells of rats (Udden and Patton, 1994) and is predicted to occur only at inhalation concentrations in excess of 200 ppm for 6-hr exposures (Corley *et al.*, 1994). The lowest concentration (0.05 mM) is very near the LOEL for pre-hemolytic changes in red blood cells of rats (M. Udden, personal communication).

In the present study, the active transport of BAA by the kidney slice was determined by comparing the uptake of BAA with and without inhibiting the active transport process. The inhibition of active transport was accomplished with either probenecid (at least three-fold excess) or by incubating the slices in an ice bath (mouse only). A two-compartment model was used to analyze the concentration time-course data utilizing data from all time points and incubation concentrations of BAA to estimate the rate constants for active transport. It was especially important to use all of the data for mice since only very small, but consistent increases in BAA uptake occurred in kidney slices not inhibited by either probenecid or ice. In both species, the majority of the uptake represented partitioning and possibly protein-binding (referred to as non-specific uptake).

The data presented here suggest that the renal active transport of BAA differs between male and female rats, young and old female rats, and between rats and mice. However, these

differences showed a strong dose-dependency. As expected, the most significant difference occurred between species. At low BAA concentrations, the rates of active transport were very similar in rats and mice with the exception that very little active transport was detected in young female mice (Figure 5a). By comparison, high BAA concentrations resulted in significantly greater rates of active transport in rats than mice for comparable gender and age groups (Figure 5b).

Within a species, differences in active transport were primarily associated with gender and not age. For rats, males were slightly more efficient in transporting BAA than females at lower concentrations (Figure 5a) as reflected by their lower  $K_m$ s (Table 1). At higher concentrations, the reverse was observed. Age-related differences in rats were confined to the females where a slightly higher capacity and lower affinity for active transport was observed in younger versus older females (Table 1). In mice, young female mice showed a very weak capacity for acid transport of BAA compared to males (Figure 5). Since no aged female mice were available to conduct this study, age comparisons were limited to the males with younger males slightly more effective than older males in actively transporting BAA.

Circulating BAA levels are impacted by the dose/dose-rate of BE as well as by the relative tissue:blood partition coefficients, protein-binding and renal active transport of BAA as described by Lee *et al.* (1995). Grant *et al.* (1999) recently compared the relative rates of hepatic BAA formation from BE via the alcohol/aldehyde dehydrogenase pathway using the same groups of animals as in this study. A rank order of BAA formation (Grant *et al.*, 1999) and renal active transport is given in Table 2. When BAA formation and renal active secretion are considered collectively, several predictions can be made. Among rats, gender- and age-related differences in toxicity could be predicted based on projected circulating levels of BAA. For

example, young male rats form BAA slower than young female and old male rats and they secrete BAA faster than young female rats, which should lead to higher circulating levels of BAA in young female and old male rats (Table 2). These data correlate well with *in vivo* toxicity data, which found that young males are less sensitive to BE-induced toxicity than females or old male rats (Ghanayem *et al.*, 1987b, 1990; Tyler, 1984).

While no *in vivo* kinetic data exist for mice, several *a priori* predictions can be made based on the rank order of BAA formation and active secretion. For example, young female mice are likely the most sensitive among the groups of mice tested as they have the highest rate of metabolism to BAA but a limited capacity to actively secrete BAA compared to both young and old male mice (Table 2). In addition, while no age-related differences were observed with respect to active secretion of BAA in young and old mice, old mice formed BAA slightly faster, which could result in slightly higher circulating levels of BAA for longer periods of time resulting in increased hemolytic potential (Table 2).

While it is clear from these studies that there are species-, gender-, and age-related differences in the renal active transport of BAA in laboratory animals, these data alone are insufficient to predict the *in vivo* internal dose of BAA following various BE exposures. However, the comparative *in vitro* rates of renal active transport and the *in vivo* studies conducted in male rats (Ghanayem *et al.*, 1990) can be used to estimate the *in vivo* rates of active transport in young and old female rats and mice of both genders, using the parallelogram approach of Reitz *et al.* (1988). Additional studies are also in progress, which evaluate the tissue partitioning and plasma protein binding of BAA as a function of species, gender and age. This information will be important in developing the next generation PBPK model capable of

describing the internal dosimetry of BE and BAA in young and old, male and female, rats and mice.

#### ACKNOWLEDGEMENTS

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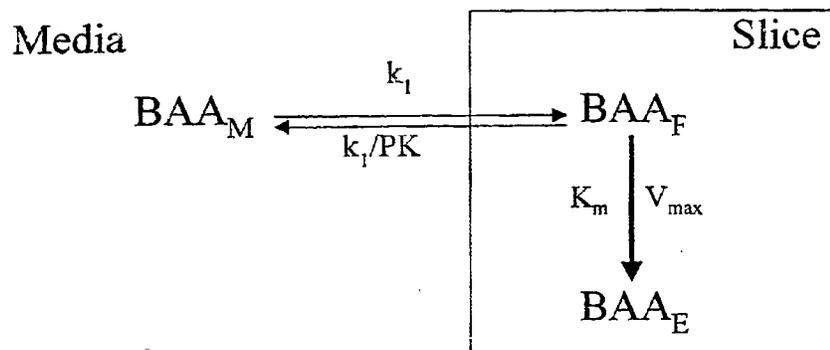
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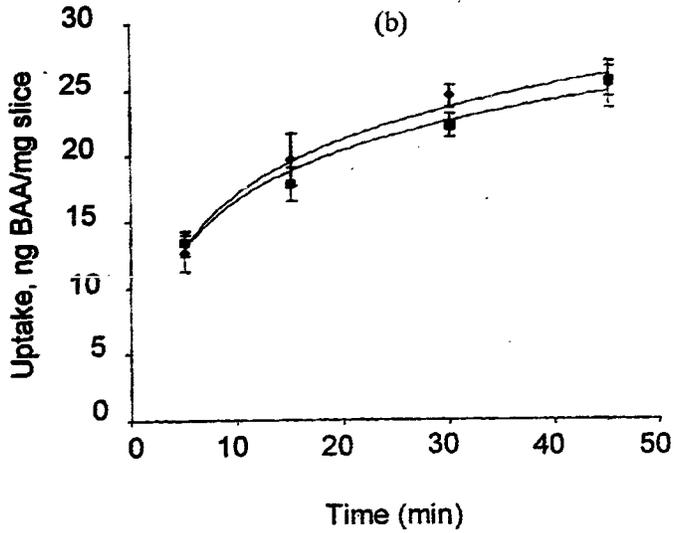
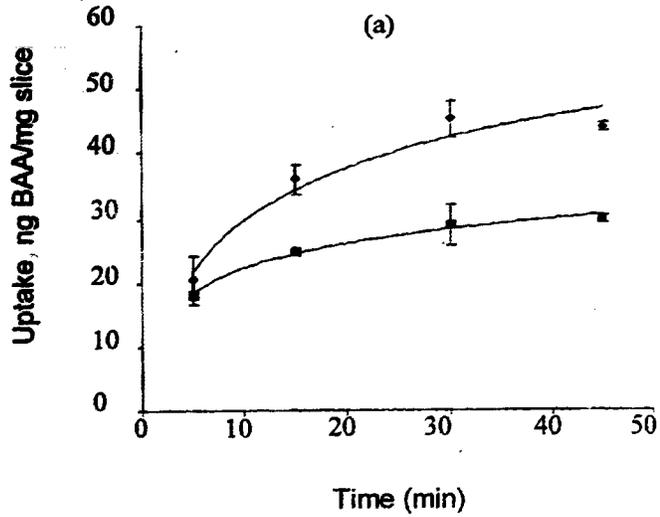
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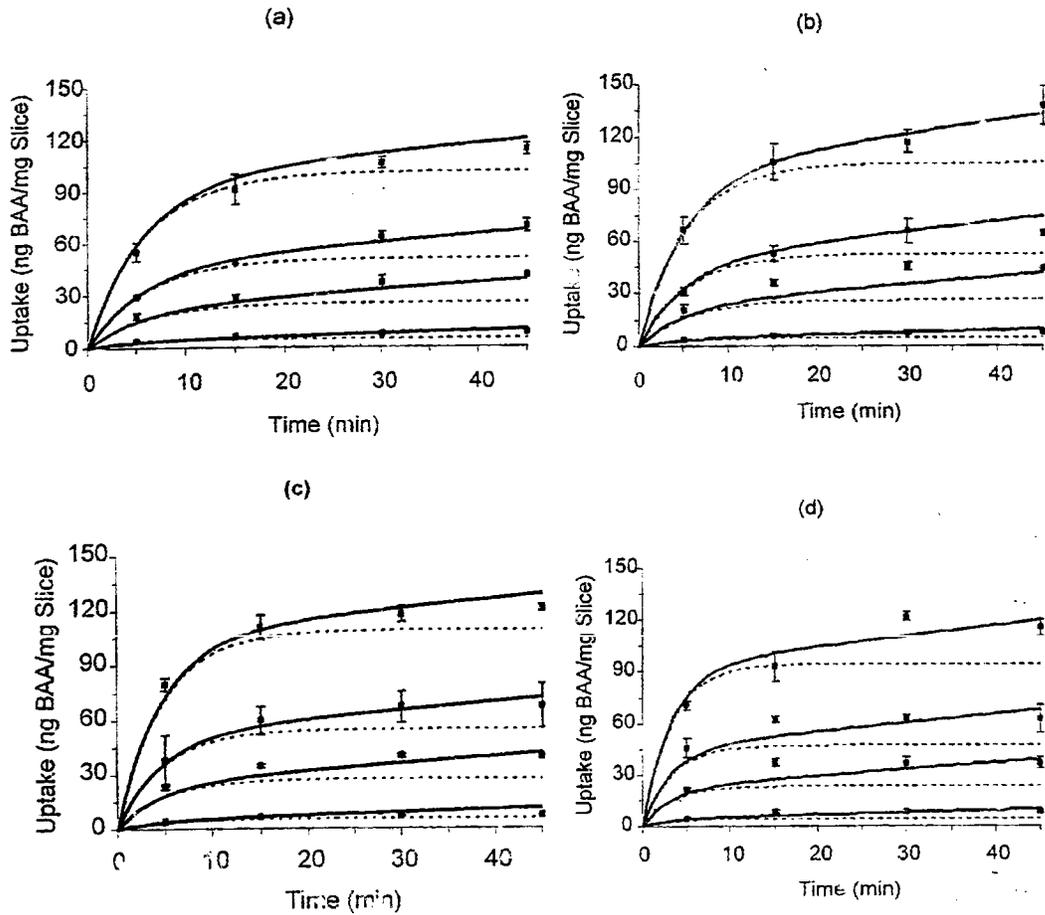
FIG. 1. Two-compartment model describing the uptake and active transport of BAA by kidney slices. PK is the kidney:media partition coefficient,  $k_1$  is the first-order transfer coefficient between media and slice,  $K_m$  and  $V_{max}$  are the Michaelis-Menten constants for renal active transport,  $BAA_M$ ,  $BAA_F$  and  $BAA_E$  are the concentrations of BAA in the media, free in the slice and actively transported in the slice, respectively.



**FIG. 2.** Uptake of BAA into (a) young female rat, and (b) young female mouse renal slices incubated in the presence of either 0.25 mM BAA alone (♦) or in combination with 1.0 mM probenecid (■). Data are means  $\pm$  SD of uptake into kidney slices (n=4).

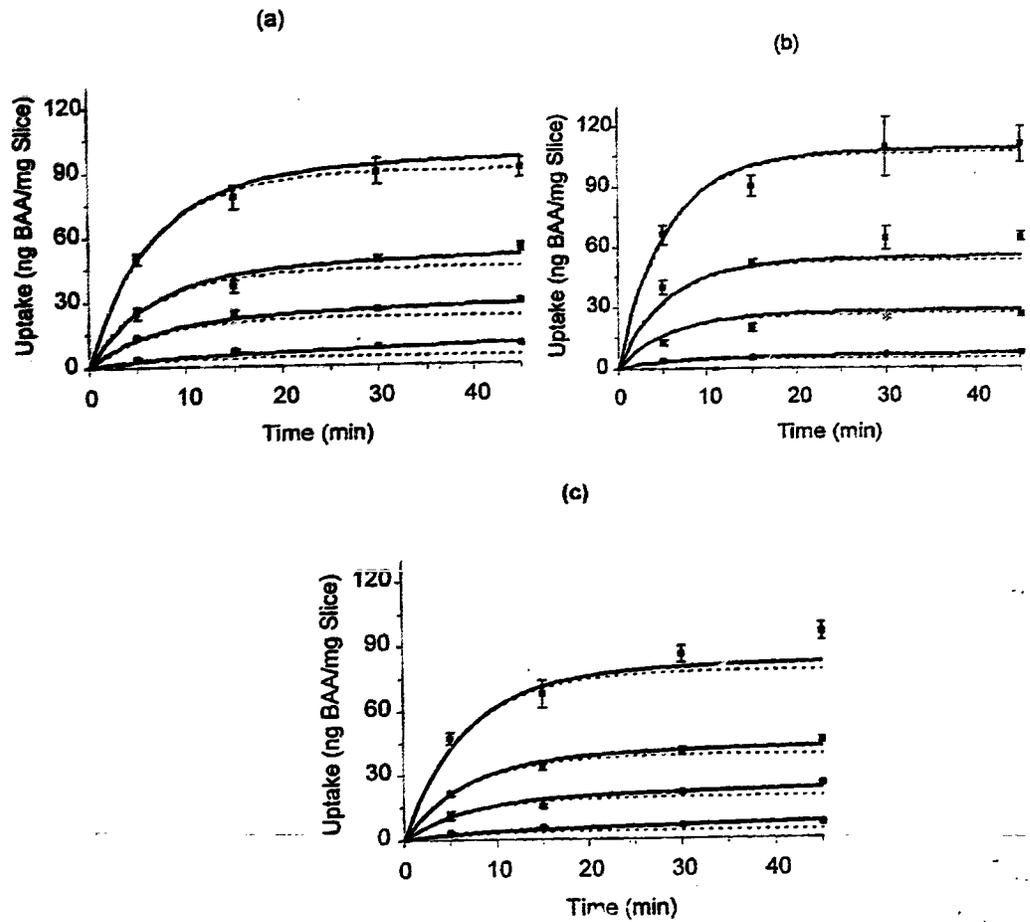


**FIG. 3.** Two-compartment model simulations (lines) and data (symbols, means  $\pm$  SD) for the uptake of BAA by (a) young male rat, (b) young female rat, (c) old male rat, and (d) old female rat kidney slices incubated without probenecid to inhibit active transport. Simulations of the non-specific uptake of BAA (BAA incubated in the presence of probenecid) are included (dotted lines) for comparison. The concentrations of BAA in the media were 0.05, 0.25, 0.5 and 1.0 mM.

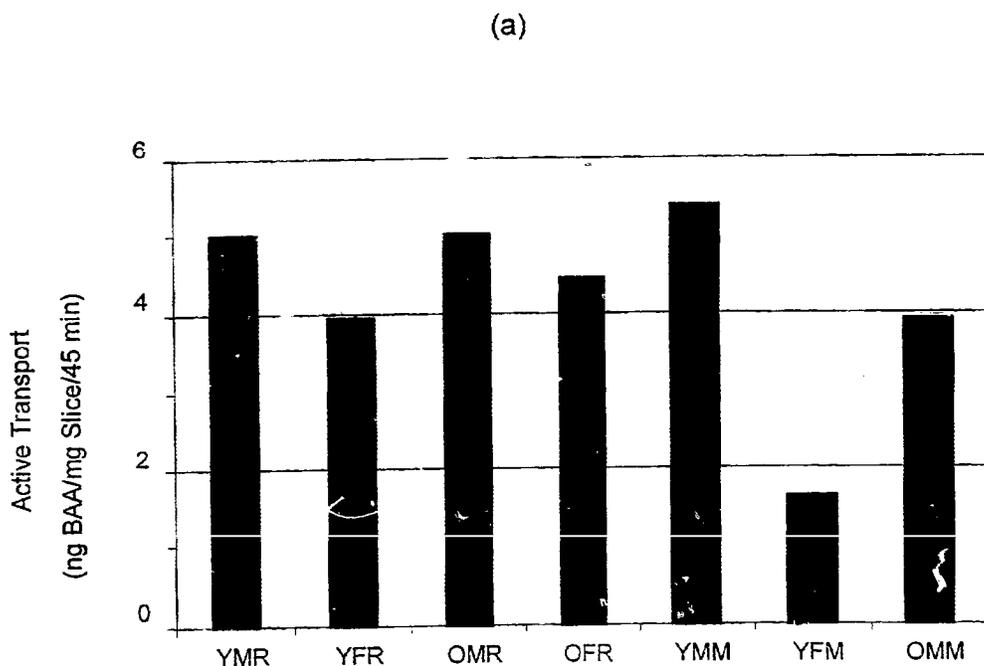


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FIG. 4. Two-compartment model simulations (lines) and data (symbols, means  $\pm$  SD) for the uptake of BAA by (a) young male mouse, (b) young female mouse and (c) old male mouse kidney slices incubated without probenecid to inhibit active transport. Simulations of the non-specific uptake of BAA (BAA incubated in the presence of probenecid) are included (dotted lines) for comparison. The concentrations of BAA in the media were 0.05, 0.25, 0.5 and 1.0 mM.



**FIG. 5.** Two-compartment model simulations of the rate of active transport in renal slices after 45 min of incubation (BAA alone – [BAA + Probenecid]) assuming the same non-specific uptake of BAA by all groups of kidney. Simulations conducted at a concentration of BAA in the media of (a) 0.05 mM and (b) 1.0 mM. YMR = young male rat, OMR = old male rat, YFR = young female rat, OFR = old female rat, YMM = young male mouse, OMM = old male mouse and YFM = young female mouse.



(b)

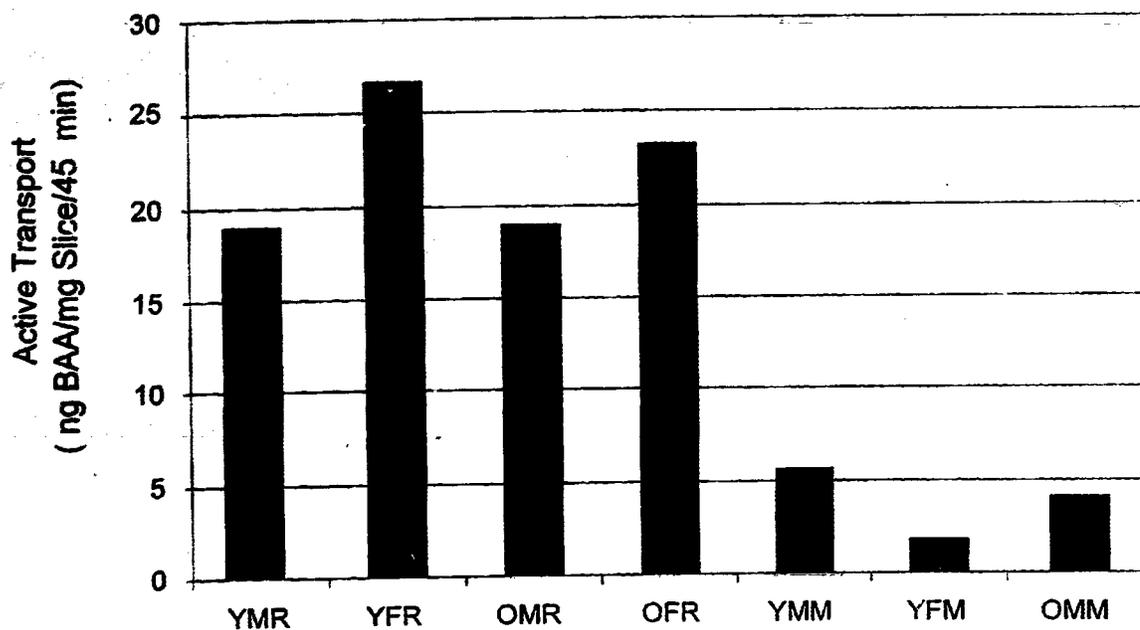


TABLE 1

**Two-Compartment Model Parameters of 2-Butoxyacetic Acid uptake  
by precision cut renal slices**

Group	$k_1$ ( $\text{min}^{-1}$ )	PK	$K_m$ (ng/mg slice)	$V_{\text{max}}$ (ng/min*mg slice)
Young male rat	0.131	0.767	12.31	0.562
Old male rat	0.176	0.827	12.31	0.562
Young female rat	0.151	0.798	32.39	0.968
Old female rat	0.220	0.719	21.18	0.758
Young male mouse	0.106	0.684	$4.96 \times 10^{-6}$	0.137
Old male mouse	0.090	0.591	$6.12 \times 10^{-6}$	0.0996
Young female mouse	0.147	0.805	$5.50 \times 10^{-6}$	0.0417

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**TABLE 2**

**Rank Order Predictions of 2-Butoxyacetic Acid Formation and its Renal Active Secretion<sup>1</sup>**

	Rat				Mouse		
	Young		Old		Young		Old
	M	F	M	F	M	F	M
BAA formation	+	++++	++++	+++	++	+++++	+++
BAA Renal Secretion	++++	++	++++	+++	+	-	+

<sup>1</sup> Rank order predictions for BAA formation (V/S ratio) (Grant *et al.*, 1999) and renal active secretion on a scale of one (+) being the lowest rate to five (+++++) for the highest rate.

**CERTIFICATE OF AUTHENTICITY**

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