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Sex Differences in the Enzymatic Hydrolysis of Acetylsalicylic Acid by Microsomes from Various Rat Tissues

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We studied the *in vitro* hydrolysis of acetylsalicylic acid (ASA) to salicylic acid (SA) catalysed by microsomal preparations from liver, kidney, small intestine and stomach mucosas and blood serum of adult female and male rats. Hepatic microsomes from male rats had the highest specific activity: 42.3 ± 6.0 nmol SA mg^{-1} min^{-1} (mean ± SEM). Kidney, intestine, stomach and serum activities were 60, 30, 14 and 0.7% with regard to the liver. In contrast, gastric microsomes from female rats showed the highest specific activity: 53 ± 22.1 nmol SA mg^{-1} min^{-1} (mean ± SEM) whereas intestine, liver, kidney and serum activities were 60, 43, 40 and 1.7% with regard to the stomach mucosa. Hepatic, renal and intestinal microsomes had a pH optimum of 5–6. Male rats had V_{max} and K_m values of 95.5, 83.4 and 29.4 nmol SA mg^{-1} min^{-1} and 2.9, 1.27 and 6.4 mM, while for female rats they were 54.8, 75.8 and 59.4 nmol SA mg^{-1} min^{-1} and 2.6, 1.35 and 3.4 mM for hepatic, renal and intestinal microsomes, respectively. Parathion inhibited the hydrolysis of ASA with an ic_{50} of 1.2×10^{-5} M for liver and kidney and 5×10^{-6} M for intestine from male rats. © **1997 John Wiley & Sons, Ltd.**
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INTRODUCTION

Since their introduction to therapeutics at the end of the last century, salicylates are drugs that have been widely used in medical practice and by the general public through self-prescription. Acetylsalicylic acid (ASA) is the salicylate most frequently used. Orally administered salicylates are absorbed rapidly, initially from the stomach but mainly from the upper small intestine. Blood levels of ASA are relatively low after oral ingestion and only 27% of the total plasma salicylates are in the acetylated form 30 min after a dose of 650 mg.[1] Pharmacokinetic studies performed in humans,[2] dogs[3] and rats[4] suggested that ASA is initially hydrolysed in the gastrointestinal tract before reaching the liver when this drug is administered by the oral route. Iwamoto *et al.*[5] even estimated that the gastrointestinal factor is relatively more important than the hepatic factor for the first-pass hydrolysis of ASA in rats. The production of salicylic acid (SA) by hydrolysis of ASA *in vitro* has been demonstrated in various tissues of several species, including humans.[6–16] This reaction is catalysed by the carboxylesterases (EC 3.1.1.1), localized mostly in the microsomal fraction of pig,[17] rat[18] and guinea pig[19] liver, and rat kidney[20] and intestine.[21] Furthermore, four of the five carboxylesterase isoenzymes purified from rat liver[14] were able to hydrolyse ASA.[22]

Sex differences of ASA hydrolysis in human serum[9] and in the pharmacokinetics of ASA in blood plasma of humans[23] have been reported. Likewise, the hydrolytic activity of rat liver carboxylesterases has shown differences related to sex.[24–29]

The rate of drug biotransformation has a decisive influence on the magnitude and duration of its pharmacological and/or toxicological effects. As many studies have been devoted to drug biotransformation by the liver, there is relatively little information about the importance of extrahepatic tissues on the overall metabolism. Even less information exists on differences of drug biotransformation related to sex. Therefore, we decided to investigate the rate of ASA hydrolysis by liver, kidney, intestine and stomach mucosas and blood serum from female and male rats. We also studied the kinetics of ASA hydrolysis as well as the pH optimum, and the inhibitory effect of parathion on the activity of various rat tissues.

EXPERIMENTAL

Chemicals

Crystalline acetylsalicylic acid, salicylic acid, 2(*N*-morpholino)ethanesulphonic acid (MES) and Trisma base were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethyl parathion (CAS no. 56382),

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98% pure, was kindly supplied by QFI Fernando Alba from the Plant Sanitary Unit (SARH); its purity was assessed by thin layer chromatography. All other chemicals were of the highest purity commercially available.

Animals

Adult albino Wistar rats 12–18 weeks old and weighing 180–200 g (6–18 females) and 180–360 g (6–15 males) were used. Rats were euthanized by cervical dislocation and the thorax and abdomen were exposed. Immediately, blood samples were withdrawn from the heart, allowed to clot and centrifuged at 1500 g for 15 min to obtain serum. The liver was perfused *in situ* with cold isotonic saline solution through the suprahepatic vein until most of the blood was eliminated; the same treatment was applied to the kidneys, perfused through the renal artery. The liver and kidneys were minced with scissors. The whole stomach and a portion of the jejunum (approximately 50 cm long) were opened, washed thoroughly with cold saline solution and the mucosa was scraped off with a spatula.

Preparation of microsomes

Hepatic, renal, gastric and intestinal microsomes were prepared by calcium precipitation.[30] All procedures were carried out at 2–4°C.

Biochemical assays

The enzymatic activity was measured at 37°C by salicylic acid determination.[31] The routine assay had 50 mM potassium phosphate (pH 7.4) or 50 mM MES (pH 5.5) in a final volume of 0.5 ml. The reaction, usually done in duplicate, was started by the addition of ASA in 10 μl of ethanol and stopped with 0.5 ml of 20% trichloroacetic acid. The precipiated protein was removed by centrifugation at 1100 *g* for 5 min and the supernatant was read at 300 nm. To calculate the specific activity, the hydrolysis rates in the absence of enzyme were deducted from the total enzymatic rates. Protein was determined with the Folin phenol reagent using bovine serum albumin as standard.[32]

Inhibition studies

Several parathion concentrations were prepared in ethanol. Equal aliquots were pipetted into glass tubes and ethanol was evaporated with a stream of nitrogen before a 30-min preincubation of the microsomal fractions with the organophosphate; then, activity was measured in buffer solution by the addition of substrate.

Statistical analysis

The significance of the difference between two mean values of enzymatic activity and of the kinetic constants (V_{max} and K_m) were calculated by two-tailed Student's *t*-test for grouped data; the lines in Fig. 2 were generated by linear regression and they were tested for parallelism; all values were obtained with the computer program PHARM/PCS41.

RESULTS

Preliminary experiments were performed to test the linearity of hydrolysis at 37°C against incubation time, substrate concentration and concentration of microsomal protein from different tissues and blood serum. The rate of hydrolysis remained linear for more than 5 min for ASA concentrations varying from 0.5 to 10 mM. Thus, we used 5-min incubation times. The hydrolysis also remained linear with protein concentrations in the ranges of 0.1–0.3 mg/ml^{-1} for hepatic, intestinal and gastric microsomes, 0.25–0.5 mg ml^{-1} for renal microsomes and 3–9 mg ml^{-1} for blood serum.

Comparison of ASA hydrolysis rates in tissues from female and male rats

Preliminary results indicated sex-related differences of ASA hydrolysis in the 12 100 *g* supernatant from liver, intestine and stomach mucosas. Therefore, we decided to investigate ASA hydrolysis by the microsomal fractions of liver, kidney, gastric and small intestine mucosas and blood serum from female and male rats. The results obtained are depicted in Table 1. In male rats, the liver had the highest activity of all tissues tested; this activity was significantly higher ($P<0.05$) than kidney, intestine, stomach and serum, whose activities were 62, 30, 14 and 0.7% with respect to liver. In contrast, the gastrointestinal mucosas from female rats showed higher mean specific activities than liver, while similar activities were found for liver and kidney; however, the gastric and intestinal activities were not significantly different ($P>0.05$) from the liver activity, due to the large dispersion of enzymatic activities showed by these mucosas. Only female rat serum had significantly higher rates ($P<0.05$) than male rat serum. Table 1 also shows that the male/female ratio of activities is higher than 1 only for liver and kidney.

Optimum pH

We also studied the microsomal hydrolytic activity of liver, kidney and intestinal mucosa from male rats at

Table 1. Sex-related differences of acetylsalicylic acid (ASA) hydrolysis by microsomal fractions of tissues and blood serum from adult female and male rats[a]

	Specific activity (nmol salicylic acid min^{-1} mg^{-1})		
Organ	Female	Male	Ratio (M/F)
Liver	22.7 ± 3.8 (15)	42.3 ± 6.0 (15)#	1.86
Kidney	21.3 ± 5.4 (13)	26.5 ± 3.0 (14)*	1.24
Intestine mucosa	31.9 ± 16.4 (18)	12.7 ± 4.0 (14)*	0.40
Stomach mucosa	53.1 ± 22.1 (18)	6.1 ± 3.2 (14)*	0.11
Blood serum	0.9 ± 0.2 (6)#*	0.3 ± 0.16 (6)*	0.33

[a]Values are mean ± SEM. The final concentration of ASA was 2 mM. The number of rats used are in parentheses. For experimental details, see text. *$P<0.05$ when comparing liver of each sex. #$P<0.05$ for female vs male.

37°C, at various pH, using 2 and 5 mM ASA. Three buffers were prepared for this purpose: 2(*N*-morpholino)ethanesulphonic acid (MES) (pH 5–6), potassium phosphate (pH 6.5–7.5) and Tris: HCl (pH 8–9) to cover the range pH 5–9. All buffers were adjusted to the same ionic strength ($\mu = 0.1$) with NaCl. In that range, the hydrolysis of 2 mM ASA was highest from pH 5–6 for the three tissues tested (Fig. 1). Similar results were obtained with 5 mM ASA and with microsomal preparations from female rats, using both ASA concentrations. Therefore, we decided to use MES pH 5.5 for the remaining experiments.

Kinetics of ASA hydrolysis by liver, kidney and intestinal microsomes

In order to find the possible source of the differences in enzymatic activity related to sex, we determined the kinetic constants of ASA hydrolysis. The activity was measured in 50 mM MES pH 5.5 at 37°C, using 0.5–8 mM ASA. Figure 2 shows the results as double reciprocal plots. The calculated maximum velocity (V_{max}) of hydrolysis and the apparent Michaelis–Menten constant (K_m) of liver microsomes were (mean ± SEM, $n = 4$): 54.8 ± 9.0 nmol SA mg^{-1} min^{-1} and 2.6 ± 0.5 mM for female rats and 95.5 ± 10.3 nmol SA mg^{-1} min^{-1} and 2.9 ± 0.43 mM for male rats; only the V_{max} and the slopes (parallelism) were significantly different ($P<0.05$). Kidney microsomes gave the following values: the V_{max} were 75.8 ± 12 and 83.4 ± 11 nmol SA mg^{-1} min^{-1} and the K_m were 1.35 ± 0.23 and 1.27 ± 0.25 mM (mean ± SEM, $n = 3$) for female and male rats, respectively (data not shown). Figure 2 also presents the results obtained with small intestine microsomes from female rats, where the V_{max} was 59.4 ± 9.0 nmol SA mg^{-1} min^{-1} and the K_m was 3.4 ± 0.4 mM (mean ± SEM, $n = 4$) compared to a V_{max} of 29.4 ± 3.5 nmol SA mg^{-1} min^{-1} and a K_m of 6.4 ± 0.61 mM (mean ± SEM, $n = 4$) for microsomes from male rats; the K_m values were significantly different ($P<0.05$), as well as the slopes ($P<0.01$), but the V_{max} values were not.

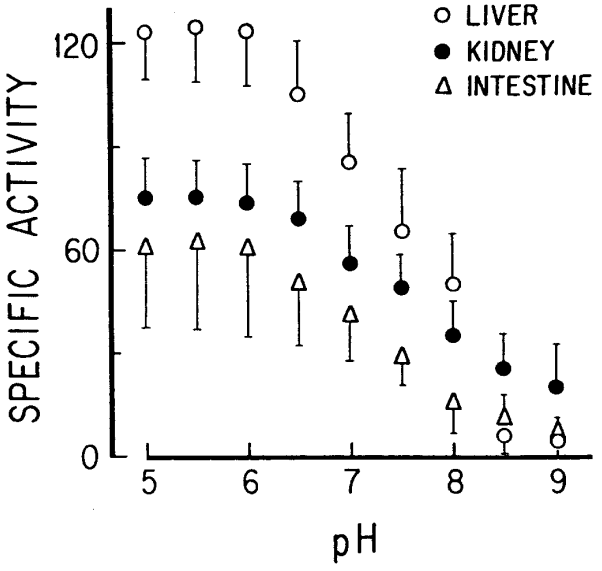

Figure 1. Effect of pH on the hydrolysis of acetylsalicylic acid (ASA). Liver, kidney and intestine microsomes from male rats were prepared as described in the Experimental. The hydrolytic activity was assayed in buffers with the same ionic strength ($\mu = 0.1$) at 37°C. The points represent the mean ± SEM ($n = 4$).

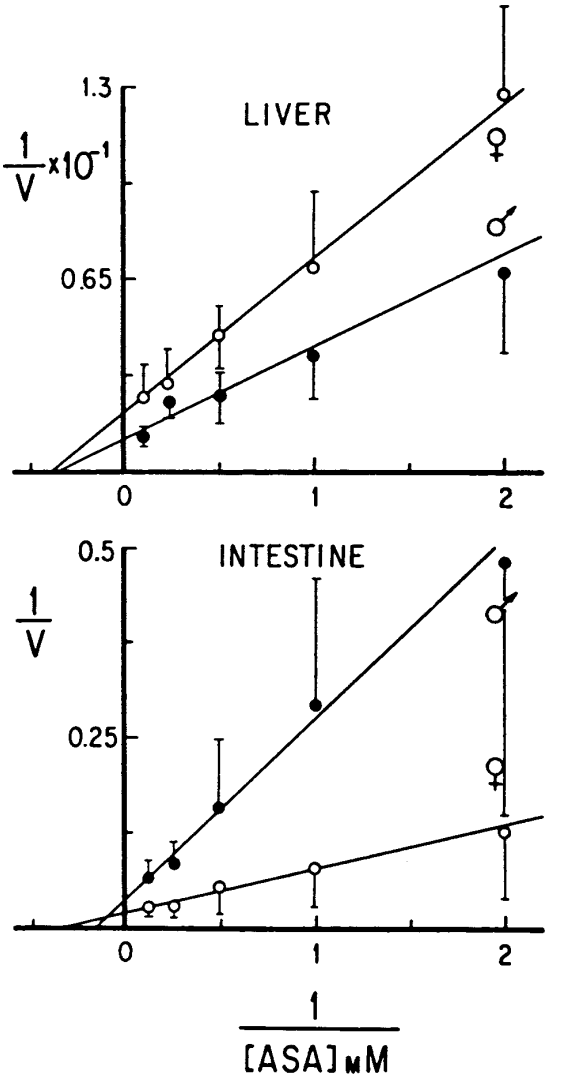

Figure 2. Double reciprocal plots of hydrolysis rates versus ASA concentrations. Liver and intestine microsomes from female and male rats were prepared as in the Experimental. The enzymatic activity was assayed in 50 mM MES (pH 5.5) at 37°C, using 0.5–8 mM ASA. The points represent the mean ± SEM ($n = 3$–4).

Parathion inhibition

The organophosphates are well-known inhibitors of esterases.[33] Because we had studied the effects of ethyl parathion on whole rats,[34,35] we used parathion to determine the concentration required for 50% inhibition (ic_{50}) of ASA hydrolysis by liver, kidney and intestine microsomes from male rats. Parathion concentrations ranged from 5×10^{-4} to 5×10^{-7} M. Preliminary results indicated that a 5-min preincubation with the inhibitor was enough to attain maximum inhibition. This preincubation time and the two washes given to the microsomal preparations kept the possible conversion to paraoxon to a minimum. The results are depicted

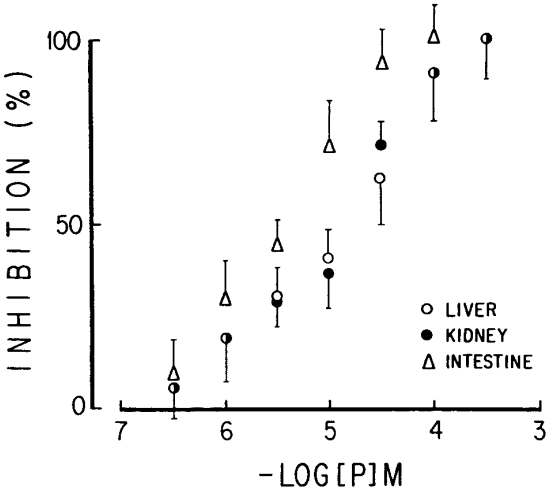

Figure 3. Inhibition of ASA hydrolysis by parathion. Semilogarithmic plots of inhibition of enzymatic activity (%) versus parathion concentrations. Liver, kidney and intestine microsomes from male rats were prepared as in the Experimental. The enzymatic activity was determined in 50 mM MES (pH 5.5) at 37°C, using 4 mM ASA. Final concentrations of parathion were from 5×10^{-4} to 5×10^{-7} M. The points represent the mean ± SEM ($n = 4$).

in Fig. 3. The calculated ic_{50} values of parathion were: 1.16×10^{-5} M for liver, 1.18×10^{-5} M for kidney and 5×10^{-6} M for intestine microsomes.

DISCUSSION

We have described differences of ASA hydrolysis by stomach, intestine and liver microsomal preparations, as well as by blood serum, from adult female and male rats. The liver of male rats showed significantly higher enzymatic activity than the other tissues tested, in agreement with previous reports.[6,10] In contrast, the mucosas of stomach and intestine from female rats had higher mean specific activities than liver but were not significantly different ($P > 0.05$). Gastric homogenates[11] and purified intestine esterases from humans[36,37] are capable of catalysing the hydrolysis of ASA. On the other hand, the activity of blood serum from female rats is 4% of the liver hydrolysis rate, whereas in male rats the serum activity is only 0.7% of the hepatic rate; the higher rate of sera from female rats is in contrast to those reported for human sera.[9] The hydrolysis of ASA by hepatic, renal and intestinal microsomes from male rats had optimum enzymatic activity at pH 5–6; these results partially agree with those described for rat liver and kidney homogenates.[16] Liver microsomes from female and male rats showed a similar apparent K_m for ASA hydrolysis (2.6 and 2.9 mM) but the V_{max} of male rats was nearly double ($P < 0.05$) that for females; these findings agree with earlier reports,[26,28] showing that adult male rats have higher amounts of the two carboxylesterases (p*I* 6.2 and 6.4) mainly involved in ASA hydrolysis.[22] There were no differences in the kinetic constants of ASA hydrolysis by kidney microsomes related to sex (data not shown). However, intestine microsomes from female rats hydrolyzed ASA twice as fast as male rat microsomes ($P > 0.05$) with approximately twice as low apparent K_m ($P < 0.05$); these results allow us to suggest that female rats might have higher amounts of the carboxylesterases involved in ASA hydrolysis than male rats. These findings also emphasize the known fact that sex regulates the expression of enzymes[27,38–40] in different organs and perhaps compensates for their role in the overall metabolism of xenobiotics. On the other hand, the sex differences in the gastrointestinal activity of enzymes could be important for prodrugs and drugs with a high presystemic rate of hydrolysis (first-pass effect), such ASA, because it will change the pharmacokinetics, with consequent pharmacological and/or toxicological implications.

Finally, the ASA hydrolytic activity of intestine microsomes from male rats is slightly more sensitive to parathion inhibition than liver and kidney microsomes; we believe that this inhibitory effect is due to parathion and not to paraoxon because the experimental conditions used were not propitious for parathion oxidation; furthermore, the ic_{50} for paraoxon is approximately 1×10^{-9} M (data not shown).

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