

## EFFECT OF PHENOBARBITAL AND SPIRONOLACTONE TREATMENT ON THE OXIDATIVE METABOLISM OF ANTIPYRINE BY RAT LIVER MICROSOMES

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The effects of pretreating rats with the inducers, phenobarbital or spironolactone, on the formation rate of the three major oxidative metabolites of antipyrine *in vitro* by hepatic microsomal fractions have been investigated. Both inducers reduced the rate of 3-methylhydroxylation of antipyrine by approximately 50%. In contrast, N-demethylation and 4-hydroxylation were enhanced 1.7-fold and 3.4-fold, respectively, in case of phenobarbital induction and 1.4-fold and 2.6-fold, respectively, following spironolactone treatment.

To elucidate the role of some cytochrome P450 isoenzymes in the production of the three major metabolites of antipyrine, the effects of form selective enzyme inhibitors on antipyrine oxidation were also studied. Troleanomycin did not alter 3-methylhydroxylation but reduced both N-demethylation and 4-hydroxylation of antipyrine in microsomes from induced rat liver. Cimetidine and chloramphenicol decreased the rate of formation of all three metabolites in microsomes from induced and uninduced animal livers as well. Chloramphenicol seemed to be the most potent inhibitor of *in vitro* antipyrine oxidation. -Methyldopa significantly enhanced the rate of formation of 4-hydroxyantipyrine and slightly reduced the rate of N-demethylation and 3-methylhydroxylation.

According to the data obtained with microsomes from uninduced rat livers, the formation of the three major metabolites of antipyrine is extensively mediated by CYP2C11/C6. In microsomes from induced animal liver, CYP2B and CYP3A may contribute to both N-demethylation and 4-hydroxylation of antipyrine.

**Key words:** antipyrine, phenobarbital, spironolactone,  $\alpha$ -methyldopa, cytochrome P-450, liver microsomes, rat

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*Abbreviations:* AP – antipyrine, HAP – 4-hydroxyantipyrine, HMAP – 3-hydroxymethylantipyrine, NORAP – norantipyrine, PB – phenobarbital, SPL – spironolactone, TAO – troleandomycin

## INTRODUCTION

Antipyrine (AP) has been extensively used as a model drug in studying the influence of genetic factors, age, diseases, drugs and environmental factors on the hepatic drug metabolizing enzyme activity in humans and laboratory animals [2, 3, 8, 20]. Three major oxidative metabolites of AP can be identified after *in vitro* microsomal incubations. N-demethylation of AP leads to the formation of norantipyrine (NORAP) and hydroxylation reactions result in 3-hydroxymethylantipyrine (HMAP) and 4-hydroxyantipyrine (HAP). All three compounds are further metabolized by conjugation *in vivo* [26]. Aromatic ring hydroxylation is an additional pathway in the biotransformation of AP leading to 4,4'-dihydroxyantipyrine. This compound can be quantified in urine after acidic hydrolysis of its conjugate since the free metabolite is very labile at pH > 2 [24, 26].

Selective changes in the formation of the three major metabolites of AP have been observed after pretreatment of rats with inducers like phenobarbital (PB), -naphthoflavone and 3-methylcholanthrene [5, 8, 11, 15, 21]. These findings and data obtained by the use of enzyme inhibitors or reconstituted monooxygenase systems suggest that several isoenzymes of cytochrome P450 catalyze the formation of each metabolite [6, 22].

To examine the role of some cytochrome P450 forms that may participate in production of the three major metabolites of AP in rats, we analyzed the effects of pretreating animals with PB or spironolactone (SPL) and studied the effects of form selective enzyme inhibitors on *in vitro* AP oxidation as well.

## MATERIALS and METHODS

### Chemicals

NADPH was purchased from Reanal (Budapest, Hungary). D-glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase and HPLC grade solvents were obtained from Merck (Darmstadt, Germany). PB, troleandomycin (TAO)

and chloramphenicol were all from Sigma (St. Louis, USA). SPL and cimetidine were purchased from Chemical Works of Gedeon Richter Ltd. (Budapest, Hungary). -Methyldopa was obtained from EGIS Pharmaceutical Ltd. (Budapest, Hungary). AP and its three metabolites were a generous gift from Professor Kálmán Róna (Semmelweis University, Budapest, Hungary).

### Treatment of animals

Male Wistar rats (200–250 g), obtained from Toxi-Coop (Budapest, Hungary) were maintained under constant heating and lighting cycles. Animals were permitted free access to food until 18 h before being killed, and water. Groups of rats were treated with PB or SPL suspension (40 mg/kg in 1% Tween 80 – water) per os, daily for three days. Control animals received an equivalent volume of vehicle (5 ml/kg) by gastric tube.

### Preparation of microsomes

Pooled microsomal fractions from 3 rat livers in each group (uninduced, PB-, or SPL-induced) were prepared as previously described [25] 24 h after the last treatment. Protein concentration was determined by the method of Lowry et al. [14].

### Characterization of microsomes

Aminopyrine N-demethylase activity was assayed by measuring formaldehyde as reported by Nash [17]. Specific activities expressed in nanomoles of the product formed per minute per mg of protein are as follows:  $12.33 \pm 0.23$  (n = 3) for microsomes from uninduced rats,  $25.29 \pm 2.74$  (n = 4) for microsomes from PB-induced rats, and  $22.92 \pm 0.40$  (n = 4) for microsomes from SPL-induced animals. Pentoxyresorufin O-dealkylation was determined by the method of Burke et al. [1]. Specific activities expressed in nanomoles of the product formed per minute per mg of protein are as follows:  $0.08 \pm 0.01$  (n = 4) for microsomes from uninduced rats,  $2.01 \pm 0.27$  (n = 12) for microsomes from PB-induced rats, and  $0.16 \pm 0.02$  (n = 3) for microsomes from SPL-induced animals.

### *In vitro* antipyrine oxidation

For the analysis of HMAP, NORAP and HAP formation, 1 mg of microsomal protein was preincubated at 37°C for 15 min in 0.8 ml of 0.1 M Tris-HCl buffer (pH 7.4) containing 2.5 mM

$\text{Na}_2\text{S}_2\text{O}_5$ , 1.25 mM NADPH, 6.25 mM  $\text{MgCl}_2$ , 6.25 mM glucose-6-phosphate and 1.25 U glucose-6-phosphate dehydrogenase in the absence or presence of form selective cytochrome P450 inhibitors at concentrations indicated in Figures 2–4. Inhibitors were dissolved in 0.1 M Tris-HCl buffer (pH 7.4) except TAO which was dissolved in ethanol, its desired amount was added into a test tube and evaporated to dryness. TAO then was redissolved in microsomal protein suspension before addition of the other components to initiate AP oxidation. AP oxidation was initiated by the addition of 200  $\mu\text{l}$  of AP to give a final concentration of 16 mM. The reaction proceeded for an additional 7.5 min before quenching with 335  $\mu\text{l}$  of 20% trichloroacetic acid. The samples were centrifuged for 10 min at 4000 rpm. After addition of 40 mg  $\text{Na}_2\text{S}_2\text{O}_5$  and 200  $\mu\text{l}$  of 4 M NaOH to the supernatant, the 100-fold excess of AP was removed by 3 repeated extractions with 10 ml of toluene. This procedure removed approximately 95% of AP. The pH of the aqueous phase was then adjusted to 7.0 by addition of 2 ml of 1 M potassium phosphate buffer (pH 6.0). After addition of 660 mg of NaCl, the samples were vortexed for at least 30 s and the metabolites were extracted into 10 ml of chloroform/ethanol 9:1 (v/v) as described by Teunissen et al. [24]. This extraction step was repeated once more with 5 ml of chloroform/ethanol 9:1 (v/v). The organic phases from the two extractions were combined and evaporated to dryness. The residue was dissolved in 100  $\mu\text{l}$  of methanol/water 1:1 (v/v) and 20  $\mu\text{l}$  were injected for HPLC analysis.

#### HPLC analysis of antipyrine metabolites

Twenty microliters of the samples were injected onto a Lichrosphere 125  $\times$  4 mm reversed phase column with particle size of 5  $\mu\text{m}$  (Merck, Darmstadt, Germany). The chromatographic equipment consisted of two Isco model 2350 pumps and an Isco V<sup>4</sup> type UV detector (Isco, Lincoln, Nebraska) set at 254 nm. The mobile phase was isocratic at a flow rate of 1.5 ml/min and composed of 13.5% acetonitrile and 0.1% triethylamine (v/v) in 0.02 M phosphate buffer, pH 7.3. When the sample contained chloramphenicol, the same mobile phase was used at pH 6.9 because of an interfering chloramphenicol metabolite. Retention times for the three metabolites of AP were 2.51 min for HMAP, 4.89 min for NORAP and 9.57 min for HAP. Unextracted standards were used to construct calibration

curves for each metabolite by measuring absorption peak area. The overall recoveries of HMAP, NORAP and HAP from spiked microsomal incubations were: 91.8, 95.6 and 76.9%, respectively. Enzyme activities were corrected for recoveries and were expressed per mg of microsomal protein per minute.

#### Statistical analysis

The data represent the means  $\pm$  SD of at least three independent determinations each assayed in triplicate. Significant difference of a value compared to the control was determined using ANOVA followed by Student's *t*-test.

## RESULTS

### Formation of the three major oxidative metabolites of AP (Fig. 1)

To saturate enzymes with AP substrate, the concentrations between 15 and 20 mM are commonly used [3, 10]. In this study, formation rates of the three major oxidative metabolites of AP were measured in liver microsomes from uninduced and PB-, or SPL-induced rats using substrate concentration of 16 mM. Metabolite formation was linear up to protein concentration of at least 1 mg/ml as illustrated for uninduced microsomes. Production of the three metabolites was also linear up to 10 min (data not shown).

### Effect of pretreatment of rats with cytochrome P450 inducers (Tab. 1)

Pretreatment of rats with PB or SPL altered the rates of formation of all three metabolites com-

Table 1. Effect of pretreatment of rats with PB or SPL on the formation rate of the three major oxidative metabolites of antipyrine *in vitro* by hepatic microsomal fractions

Microsomes	Specific activity (nmole of product / mg $\times$ min)		
	HMAP	NORAP	HAP
Uninduced	1.55 $\pm$ 0.10	1.04 $\pm$ 0.12	0.81 $\pm$ 0.12
PB-induced	0.85 $\pm$ 0.07***	1.73 $\pm$ 0.23**	2.78 $\pm$ 0.43***
SPL-induced	0.87 $\pm$ 0.17***	1.50 $\pm$ 0.24**	2.08 $\pm$ 0.47**

Values are means  $\pm$  SD (n = 4–5). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with the values obtained with microsomes from uninduced rats

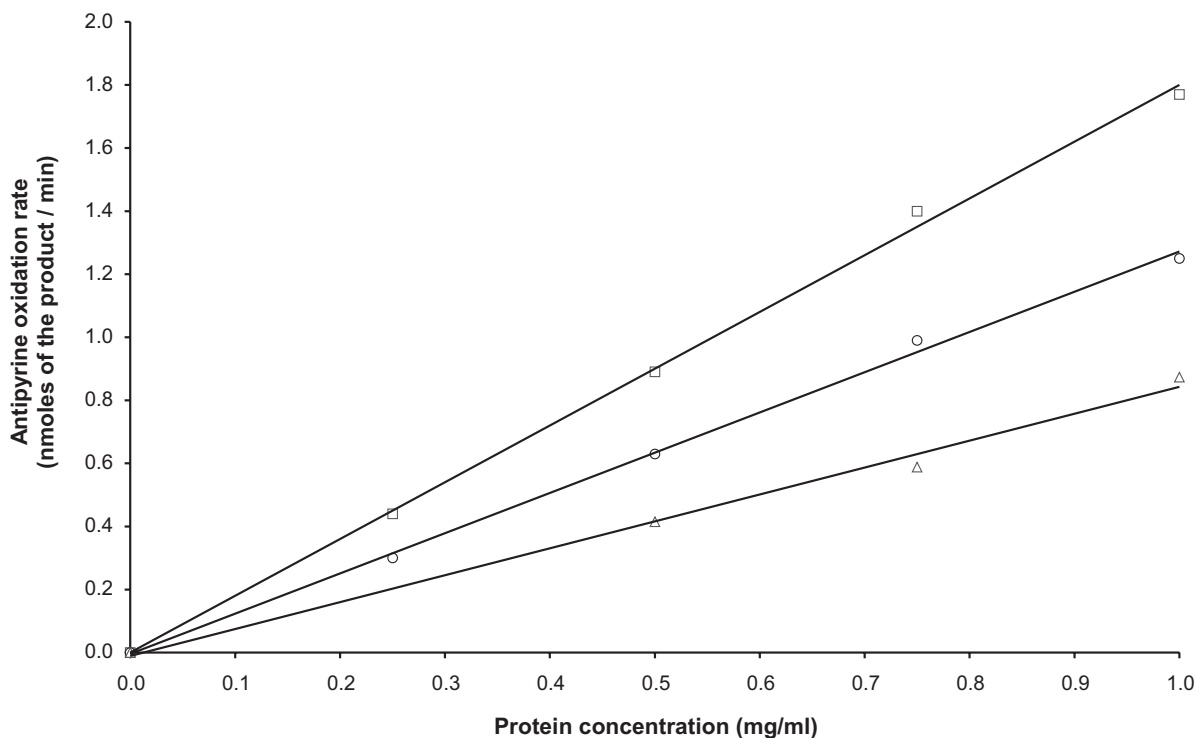


Fig. 1. Effect of microsomal protein concentration on the production of HMAP – □, NORAP – ○, and HAP – △, in liver microsomes from uninduced rats

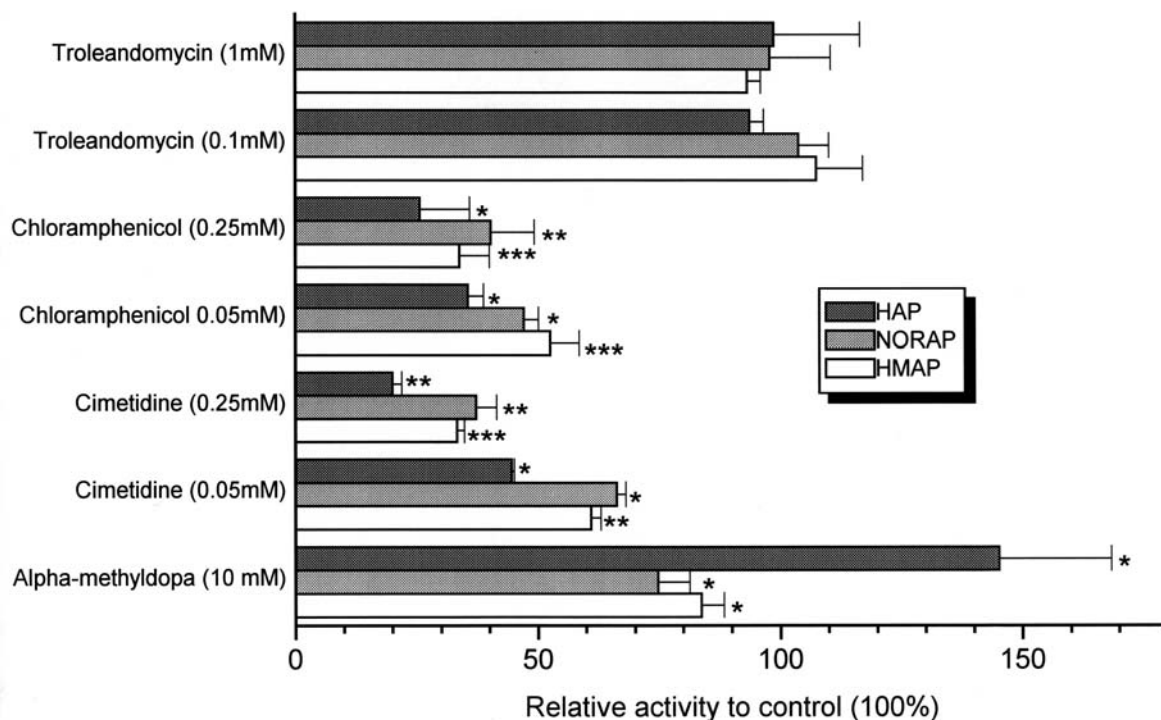


Fig. 2. Inhibition of AP metabolism in liver microsomes from uninduced rats. Incubations were performed for 7.5 min in the presence of 1 mg/ml of microsomal protein at 16 mM AP. For control activities (nmole/min/mg of protein) in the absence of inhibitors see Table 1. Mechanism-based inactivators were preincubated with microsomes and 1.25 mM NADPH (plus NADPH generating system) for 15 min at 37°C in the absence of AP. Each bar represents the mean of 3–5 determinations ± SD \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001 compared with values obtained in the absence of inhibitor

pared to the values obtained with microsomes from uninduced animals. Both inducers reduced the rate of 3-methylhydroxylation of AP by approximately 50% and this was accompanied by increases in the rates of N-demethylation and 4-hydroxylation as well. PB pretreatment increased NORAP formation 1.7-fold and HAP formation 3.4-fold. Both pathways were also susceptible to SPL induction, however SPL was less effective than PB.

### **Effect of preincubation of microsomes with mechanism-based cytochrome P450 inhibitors in the presence of NADPH** (Fig. 2, 3, 4)

#### ***Inhibition of AP metabolism by TAO***

Incubation of microsomes from uninduced rats in the presence of TAO did not alter AP metabolism (Fig. 2). In contrast, TAO reduced both N-demethylation and 4-hydroxylation of AP in microsomes from PB or SPL-induced rats causing similar extent of inhibition in NORAP production in these microsomes. HAP formation rate was reduced to 66% of the control in microsomes from PB-induced rats whereas much lower enzyme activity (32% of control) was observed in microsomes from SPL-induced animals (Fig. 3, 4).

#### ***Inhibition of AP metabolism by cimetidine***

Cimetidine at 50  $\mu$ M decreased the rate of formation of HMAP by approximately 37% compared to control values in all types of microsomes. Similarly, N-demethylation was also inhibited in microsomes from both uninduced and induced rats. 4-Hydroxylation of AP was slightly but not significantly reduced in the presence of 50  $\mu$ M cimetidine in microsomes from PB-induced animals whereas 46% and 62% of control HAP formation were measured in microsomes from uninduced or SPL-induced rats, respectively (Fig. 2, 3, 4). Cimetidine at 250  $\mu$ M caused 50% inhibition in microsomes from PB-induced rats and only 23% inhibition in microsomes from SPL-induced animals compared to the values obtained at 50  $\mu$ M cimetidine (Fig. 3, 4).

#### ***Inhibition of AP metabolism by chloramphenicol***

Chloramphenicol significantly inhibited formation rates of all three AP metabolites in micro-

somes from uninduced and induced rats as well (Fig. 2, 3, 4).

#### ***Effect of $\alpha$ -methyldopa on AP metabolism***

-Methyldopa slightly but significantly decreased the rate of formation of HMAP and NORAP in microsomes from uninduced rats (Fig. 2). In microsomes from PB-, or SPL-induced animals only the N-demethylation of AP was reduced. Formation rate of HAP was significantly increased by -methyldopa in microsomes from uninduced as well as PB-, or SPL-induced animals (Fig. 3, 4).

## **DISCUSSION**

Analysis of product formation before and after induction should provide some information on the relative contribution of different forms of cytochrome P-450 to the formation of the metabolite. HMAP was produced to the greatest extent by microsomes from uninduced rats. Both inducers caused significant reduction in the rate of formation of this metabolite suggesting that participation of CYP2B which has elevated level in PB-induced rat liver microsomes and contribution of CYP3A which has elevated level in both PB- and SPL-induced animals [9, 12], may not be substantial in formation of HMAP. The reduction observed in HMAP production can be at least partially attributed to the suppression of some CYP2C isoenzymes. Suppression of CYP2C isoenzymes was observed by use of structurally diverse enzyme inducers [18, 27]. Both N-demethylation and 4-hydroxylation of AP were enhanced in microsomes from induced rats indicating that in these microsomes CYP3A and CYP2B might be involved in the formation of NORAP and HAP among other CYP isoenzymes also induced.

The effect of three well-documented inhibitors and -methyldopa, an antihypertensive drug, on metabolite formation has also been studied with microsomes from PB- or SPL-induced rats as well as control animals. These inhibitors have been previously shown to offer selectivity for one or more isoenzymes. TAO is considered as specific inhibitor for CYP3A [16]. Cimetidine at low concentration (50  $\mu$ M) inhibits CYP2C11/C6 whereas at higher doses, it also inhibits CYP3A and CYP2B [4, 13]. The reduction of metabolite formation rate in the presence of chloramphenicol must be inter-

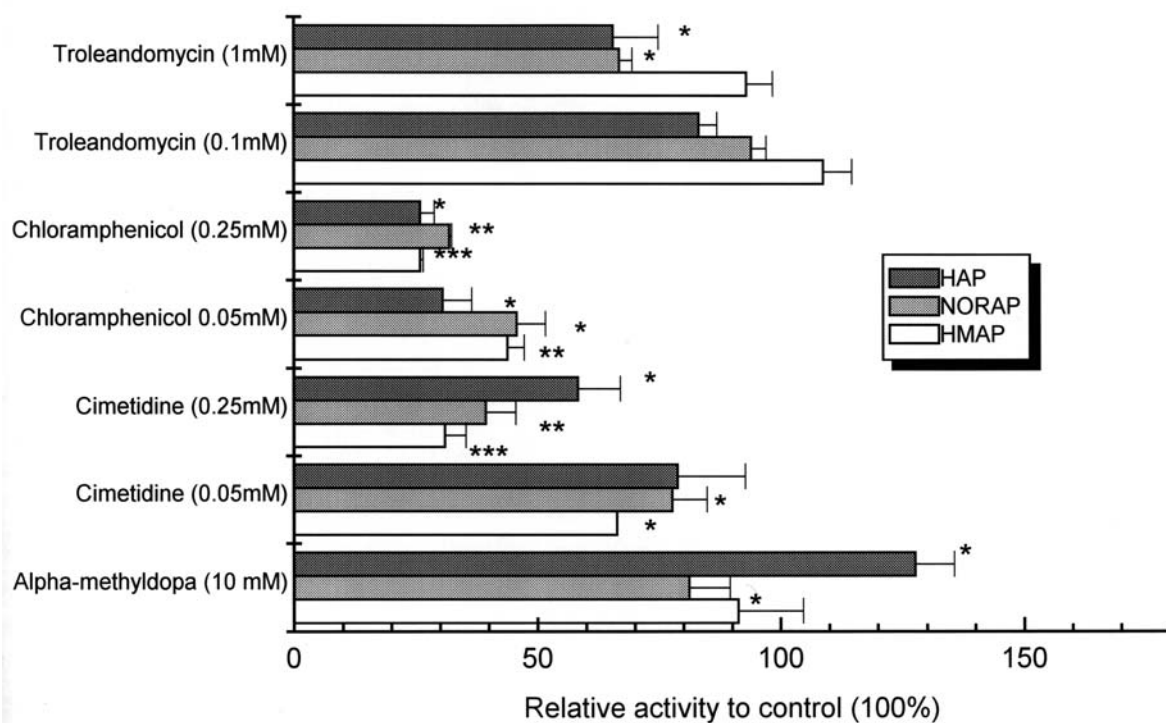


Fig. 3. Inhibition of AP metabolism in liver microsomes from PB-induced rats. For assay conditions see legend for Figure 2

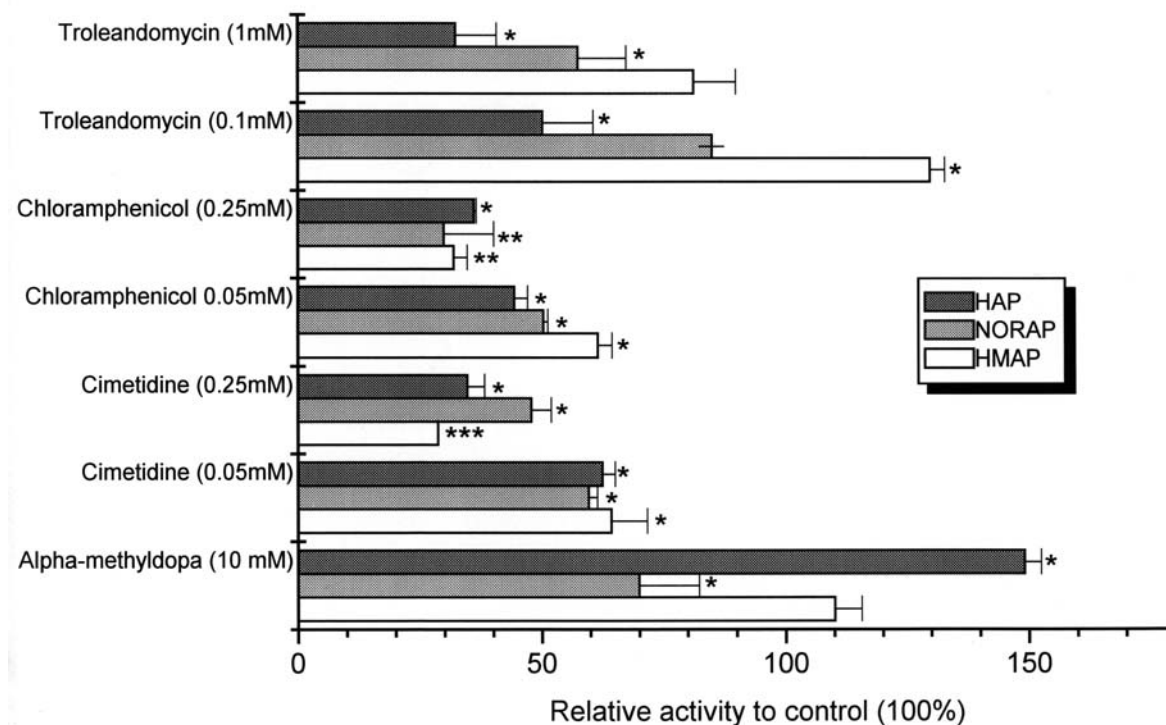


Fig. 4. Inhibition of AP metabolism in liver microsomes from SPL-induced rats. For assay conditions see legend for Figure 2

preted cautiously because this compound inactivates not only CYP2B but also CYP3A and CYP2C11 although the rate constant of inactivation for CYP2B is five-times higher than that of the other isoenzymes mentioned. [23]. Results obtained with inhibitors are expressed as percentage of control values obtained in the absence of inhibitors (Tab. 1) and are shown in Fig. 2, 3, 4.

Enzyme inhibition experiments with TAO suggest no significant contribution of CYP3A to the formation of any of the AP metabolites in microsomes from uninduced rats. HMAP formation was not inhibited either in microsomes from induced or uninduced rats making substantial participation of CYP3A unlikely in this reaction. In contrast, reduction of NORAP and HAP formation by TAO in microsomes from PB- or SPL-induced rats indicates involvement of CYP3A in AP metabolism in these microsomes.

Incubations that include cimetidine at 50  $\mu$ M should provide selectivity for CYP2C11/C6. The results indicate involvement of CYP2C11/C6 in forming HMAP, NORAP and HAP as well. Similar conclusion was also drawn by Nakagawa et al. [15] who reported that the male specific cytochrome P-450 was capable of synthesizing all three main metabolites of AP. It was shown that raising cimetidine concentration above 50  $\mu$ M resulted in the inhibition of CYP3A and CYP2B in addition to inhibition of CYP2C11/C6 whereas activity of other isoenzymes was not altered [4]. In our study, when cimetidine concentration was increased, N-demethylation in microsomes from PB-induced rats seemed to be much more susceptible to further inhibition than that in microsomes from SPL-induced animals. The CYP3A activity as judged by the rate of aminopyrine N-demethylation was not different in the case of PB or SPL induction. This result and the form selective inhibition pattern of cimetidine suggest that NORAP formation in microsomes from PB-induced rats can be catalyzed by CYP2B in addition to CYP3A and CYP2C11/C6.

Chloramphenicol is a form selective inactivator for CYP2B, CYP3A and CYP2C11. Both N-demethylation and 4-hydroxylation of AP seemed to be more sensitive to 50  $\mu$ M chloramphenicol than to 50  $\mu$ M cimetidine in microsomes from PB-induced rats compared with the sensitivity observed in microsomes from SPL-induced animals (Fig. 3, 4). This finding in addition to the results showing that a) PB and SPL induction resulted in the same in-

crease in CYP3A activity (for data see Materials and Methods) but resulted in more than an order of magnitude higher CYP2B (pentoxyresorufin O-dealkylase) activity in case of PB induction, and considering that b) cimetidine at 50  $\mu$ M decreased the NORAP and HAP formation in microsomes from PB-induced rats to a lesser extent than in SPL-induced animals indicate the involvement of CYP2B in NORAP and HAP formation in microsomes from PB-induced rats.

-Methyldopa an antihypertensive drug that can be prescribed even in pregnancy was reported to inhibit aminopyrine N-demethylation at the millimolar concentration range [7]. This reaction is mediated predominantly by CYP3A in microsomes from PB-induced rats [19]. Unexpectedly, formation rate of HAP was significantly increased by -methyldopa in microsomes from uninduced as well as PB-, or SPL-induced animals. Inhibition of further oxidation of HAP could explain this finding. After *in vivo* AP treatment, a fourth major metabolite was also isolated from urine in quantity of 10–15% of the dose [24, 26]. The compound 4,4'-dihydroxy-AP is unstable unless it is conjugated by sulphotransferase in rats and that is why the formation of this metabolite cannot be assessed in microsomal incubations lacking cofactors for conjugation reactions.

-Methyldopa may inhibit the enzyme(s) catalysing formation of 4,4'-dihydroxyAP from HAP bringing about enhancement of HAP level.

Comparing our data with the results obtained with the use of human microsomes [6, 22], there is a similarity in respect of HMAP production. Both in human and rat microsomes CYP2C isoenzymes participate in the formation of HMAP whereas there is no evidence suggesting contribution of CYP3A. HAP production was catalyzed by CYP2C isoenzymes in microsomes from uninduced as well as induced rats, in contrast no involvement of CYP2C was found in human microsomes.

In conclusion, the changes in formation rates of AP metabolites caused by pretreatment of rats with cytochrome P450 inducers PB or SPL and those caused *in vitro* by form selective inhibitors suggest that in microsomes from uninduced rats, the formation of all three metabolites of AP was effectively catalyzed by CYP2C11/C6. These isoenzymes could also participate in the production of all three metabolites in microsomes from PB-, or SPL-induced rats as well. In microsomes from PB-induced animals, CYP2B and CYP3A, in addition to

CYP2C11/C6, could be involved in the formation of both NORAP and HAP. Similarly, in microsomes from SPL-induced rats, CYP3A probably contributed to production of these metabolites of AP. The finding of this and many other studies concerning the cytochrome P450 enzyme system, that multiple enzymes are able to catalyze the formation of a metabolite, may explain that occurrence of severe drug-drug interactions is relatively rare.

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