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EFFECTS OF PHENOTHIAZINE NEUROLEPTICS ON THE RATE OF CAFFEINE DEMETHYLATION AND HYDROXYLATION IN THE RAT LIVER

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The primary metabolic pathways of caffeine are 3-N-demethylation to paraxanthine (CYP1A2), 1-N-demethylation to theobromine and 7-N-demethylation to theophylline (CYP1A2 and other enzymes), and 8-hydroxylation to 1,3,7-trimethyluric acid (CYP3A). The aim of the present study was to investigate the influence of phenothiazine neuroleptics (chlorpromazine, levomepromazine, thioridazine, perazine) on cytochrome P-450 activity measured by caffeine oxidation in rat liver microsomes. The obtained results showed that all the investigated neuroleptics competitively inhibited caffeine oxidation in the rat liver, though their potency to inhibit particular metabolic pathways was not equal. Levomepromazine exerted the most potent inhibitory effect on caffeine oxidation pathways, the effect on 8-hydroxylation being the most pronounced. This indicates inhibition of CYP1A2 (inhibition of 3-N- and 1-N-demethylation; K_i = 36 and 32 μM, respectively), CYP3A2 (inhibition of 8-hydroxylations; K_i = 20 μM), and possibly other CYP isoenzymes (inhibition of 7-N-demethylation; K_i = 58 μM) by the neuroleptics. The potency of inhibition of caffeine oxidation by perazine was similar to levomepromazine. Thioridazine was a weeker inhibitor of caffeine 3-N- and 7-N-demethylation, while chlorpromazine was weaker in inhibiting caffeine 1-N- and 7-N-demethylation, compared to levomepromazine. In summary, the obtained results showed that all the investigated neuroleptics had a broad spectra of CYP inhibition in the rat liver. The isoenzymes CYP1A2 and CYP3A2 were distinctly inhibited by all the investigated neuroleptics, while other CYP isoenzymes (CYP2B and/or 2E1) by perazine and levomepromazine. The CYP3A2 inhibition was most pronounced. (K_i = 20–40 μM).

Key words: *caffeine oxidation, rat, cytochrome P-450 activity, chlorpromazine, levomepromazine, thioridazine, perazine*

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INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is extensively metabolized in humans and laboratory animals. The primary metabolic pathways of caffeine are 3-N-demethylation to paraxanthine (1,7-dimethylxanthine), 1-N-demethylation to theobromine (3,7--dimethylxanthine), and 7-N-demethylation to theophylline (1,3-dimethylxanthine) [3, 4, 9, 17, 18, 19]. Caffeine is a marker drug for testing the activity of CYP1A2 (3-N-demethylation) in humans and rats. Moreover, it may be also considered as a relatively specific substrate of CYP3A (8-hydroxylation). In the case of 1-N- and in particular 7-N-demethylation of caffeine, apart from CYP1A2, other cytochrome P-450 isoenzymes play a significant role.

Phenothiazine neuroleptics are very often combined with other psychotropics (antidepressants, antianxiety drugs or carbamazepine) that are metabolized by different cytochrome P-450 isoenzymes. Therefore, the knowledge of interaction of phenothiazines with the cytochrome is of a great practical value, since it allows to predict their metabolic/pharmacokinetic interaction with other drugs. During phase I of their metabolism, neuroleptics which are phenothiazine derivatives undergo mainly sulfoxidation in a thiazine ring, aromatic hydroxylation and N-demethylation in a side chain. It has been shown that isoenzyme CYP2D6 contributes to the metabolism of phenothiazines in humans. Clinical studies with perphenazine have demonstrated that the disposition of this neuroleptic is related to the polymorphic hydroxylation of debrisoquine, indicating that CYP2D6 is involved in its metabolism [10]. Recent studies have shown that CYP2D6 is a main enzyme catalyzing 7-hydroxylation of chlorpromazine, the reaction being partially catalyzed by CYP1A2 [26]. Moreover, 2-sulfoxidation of thioridazine in the thiomethyl substituent and 5-sulfoxidation in a thiazine ring are governed by CYP2D6 [5, 20, 25]. In contrast, sulfoxidation of chlorpromazine in a thiazine ring was catalyzed by the subfamily CYP3A, as was shown *in vitro* [8]. CYP3A4 and CYP2C9 were identified as the major enzymes mediating perazine N-demethylation [23]. On the other hand, phenothiazine neuroleptics have been shown to inhibit competitively CYP2D6, and some of them (fluphenazine and perphenazine) also CYP1A2 [22] in human liver microsomes. In rats, the N-demethylation of promazine, perazine and thioridazine seemed to be catalyzed by the isoenzymes CYP2D1, CYP2B2 and CYP1A2 (CYP1A2 does not refer to promazine). The 5-sulfoxidation of these drugs might be mediated by different isoenzymes, e.g. CYP2D1 (promazine and perazine), CYP2B2 (perazine) or CYP1A2 (thioridazine). The 2-sulfoxidation of thioridazine could be catalyzed by CYPs 2D1, 2B2 and 1A2 [11].

The aim of the present study was to investigate the influence of phenothiazine neuroleptics with different chemical structures on cytochrome P-450 activity measured by caffeine oxidation in rat liver microsomes.

MATERIALS and METHODS

Chemicals

Chlorpromazine and thioridazine (hydrochlorides) were provided by Polfa (Jelenia Góra, Poland), perazine (dimaleate) was obtained from Labor (Wrocław, Poland), levomepromazine (maleate) from Egyt (Budapest, Hungary). Caffeine and its metabolites, NADP, DL-isocitric acid (trisodium-salt) and isocitric dehydrogenase were purchased from Sigma, St. Louis, USA. All organic solvents with HPLC purity were supplied by Merck, Darmstadt, Germany.

Animals

The experiment was carried out on male Wistar rats (230–260 g) kept under standard laboratory conditions. Liver microsomes were prepared by differential centrifugation in 20 mM Tris/KCl buffer (pH = 7.4) including washing with 0.15 M KCl, according to a conventional method.

In vitro studies into caffeine oxidation in rat liver microsomes

The *in vitro* metabolic studies were carried out at linear dependence of the metabolite formation on time, and protein and substrate concentrations. Pooled liver microsomes from six control rats were used. Each sample was prepared in duplicate. The rates of 3-N-, 1-N- and 7-N-demethylation and 8-hydroxylation of caffeine (caffeine concentrations: 100, 400, 800 nmol/ml) were assessed in the absence or presence of one of the neuroleptics added *in vitro* (neuroleptic concentrations: 200–800 nmol/ml). Incubations were carried out in a system containing liver microsomes (ca. 1 mg of protein/ml), phosphate buffer (0.1 M, pH = 7.4), $MgCl_2 \times 6\ H_2O$

(6 mM), NADP (1.2 mM), DL-isocitric acid (6 mM) and isocitric dehydrogenase (1.2 U/ml). The final incubation volume was 0.5 ml. After a 2-min preincubation, the reaction was started with the addition of NADPH generating system and the incubation lasted for 50 min. Afterwards, the reaction was stopped by adding 350 µl of 2% $ZnSO_4$ and 25 µl of 2 M HCl.

Determination of caffeine and its oxidized metabolites

Caffeine and its four oxidized metabolites were assessed using the HPLC method [14] adapted from Rasmussen and Brøsen [21]. After incubation the samples were centrifuged for 10 min at 2000 × g. A water phase containing caffeine and its metabolites was extracted with 6 ml of organic mixture consisting of ethyl acetate and 2-propanol (8:1, v/v). The residue obtained after evaporation of microsomal extract was dissolved in 100 µl of the mobile phase described below. An aliquot of 20 µl was injected into the HPLC system. A Merck-Hitachi chromatograph, "LaChrom" (Darmstadt, Germany), equipped with an UV detector was used. The analytical column (Supelcosil LC-18, 15 cm × 4.6 mm, 5 µm) was from Supelco (Bellefonte, USA). The mobile phase consisted of 0.01 M acetate buffer (pH = 3.5) and methanol (91:9, v/v). The flow rate was 1 ml/min (0–26.5 min) followed by 3 ml/min (26.6–35 min). The column temperature was maintained at 30°C. The absorbance of caffeine and its metabolites was measured at a wavelength of 254 nm. The compounds were eluted in the following order: theobromine (9.7 min), paraxanthine (15.8 min), theophylline (16.9 min), 1,3,7-trimethyluric acid (23.4 min), caffeine (30.5 min). The calculated recovery, intra-day precision, inter-day reproducibility and accuracy were similar to the respective values obtained by Rasmussen and Brøsen [21].

The obtained results were evaluated using Dixon analysis [16].

RESULTS and DISCUSSION

Literature data indicate that caffeine is a marker drug for testing the activity of CYP1A2 (3-N-demethylation) in humans and rats. Moreover, it seems also to be a relatively specific substrate of

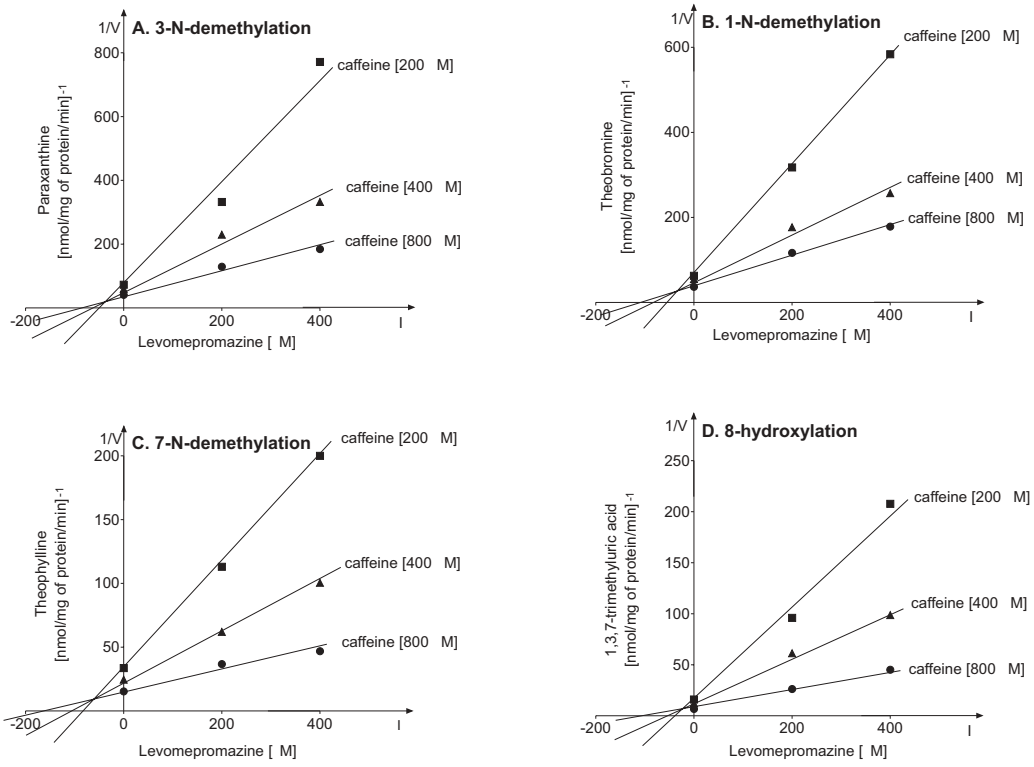

Fig. 1. The influence of levomepromazine on the metabolism of caffeine in rat liver microsomes (Dixon plots). Kinetics of the inhibition of caffeine 3-N-demethylation (A); 1-N-demethylation (B); 7-N-demethylation (C); 8-hydroxylation (D). V = velocity of the reaction, I = concentration of inhibitor

CYP3A (8-hydroxylation). In the case of 1-N- and, in particular, 7-N-demethylation of caffeine, apart from CYP1A2, other CYP isoenzymes play a considerable role, probably CYP2B and/or CYP2E1 [3, 24]. Therefore, caffeine has been chosen for our studies to show interactions of phenothiazine neuroleptics with cytochrome P-450 isoenzymes.

The obtained results showed that all the investigated neuroleptics competitively inhibited caffeine oxidation in the rat liver, though their potency to inhibit particular metabolic pathways was not equal. Dixon analysis of caffeine metabolism carried out on control liver microsomes, in the absence and presence of the neuroleptics showed that levomepromazine exerted the most potent inhibitory effect on caffeine oxidation pathways (Fig. 1 a, b, c, d), the effect on 8-hydroxylation being the most pronounced (Tab. 1). This indicates inhibition of CYP1A2 (inhibition of 3-N- and 1-N-demethylation), CYP3A2 (inhibition of 8-hydroxylations),

Table 1. The influence of phenothiazine neuroleptics on the metabolism of caffeine. The presented inhibition constants (K_i) for competitive inhibition of particular metabolic pathways were calculated using Dixon analysis

Neuroleptics (inhibitors)	Inhibition of caffeine metabolism			
	Paraxanthine (caffeine 3-N-demethylation) K_i [μM]	Theobromine (caffeine 1-N-demethylation) K_i [μM]	Theophylline (caffeine 7-N-demethylation) K_i [μM]	1,3,7-trimethyluric acid (caffeine C-8-hydroxylation) K_i [μM]
Chlorpromazine	48	76	123	40
Levomepromazine	36	32	58	20
Thioridazine	73	47	100	40
Perazine	52	50	52	32

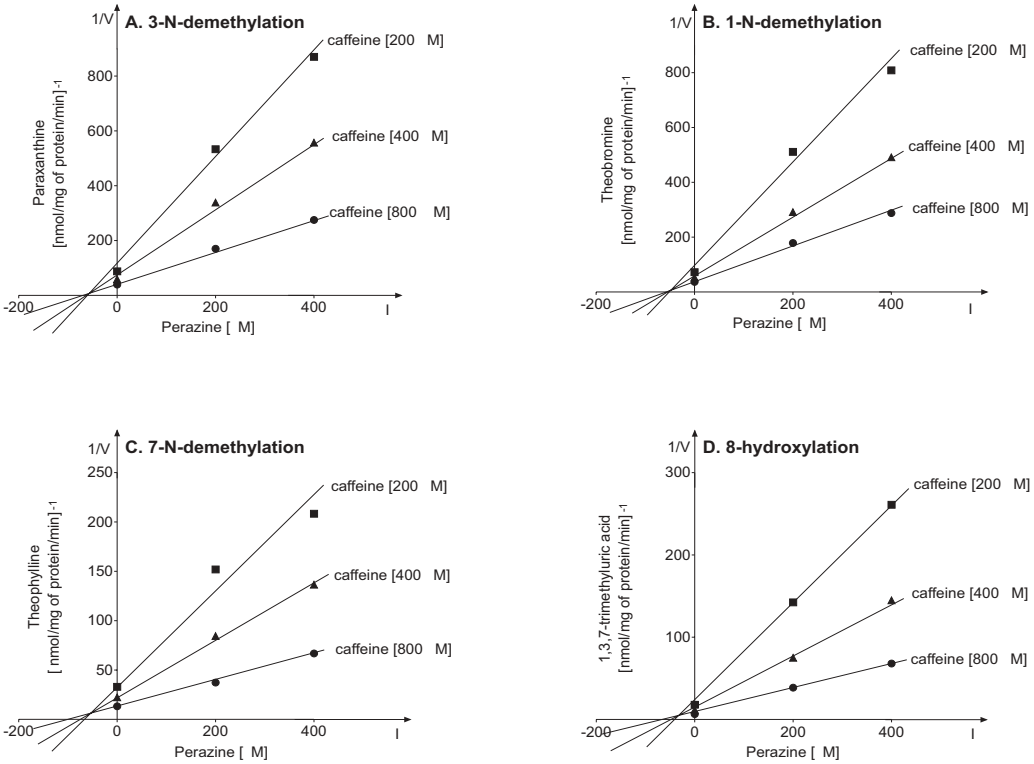

Fig. 2. The influence of perazine on the metabolism of caffeine in rat liver microsomes (Dixon plots). Kinetics of the inhibition of caffeine 3-N-demethylation (A); 1-N-demethylation (B); 7-N-demethylation (C); 8-hydroxylation (D). V = velocity of the reaction, I = concentration of inhibitor

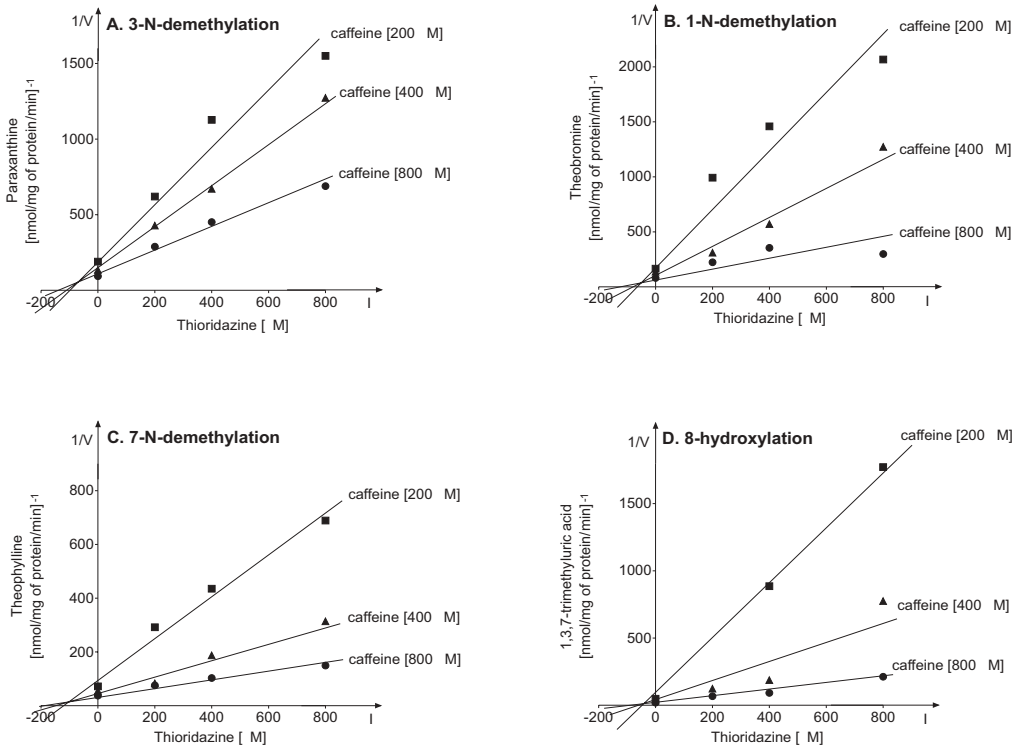

Fig. 3. The influence of thioridazine on the metabolism of caffeine in rat liver microsomes (Dixon plots). Kinetics of the inhibition of caffeine 3-N-demethylation (A); 1-N-demethylation (B); 7-N-demethylation (C); 8-hydroxylation (D). V = velocity of the reaction, I = concentration of inhibitor

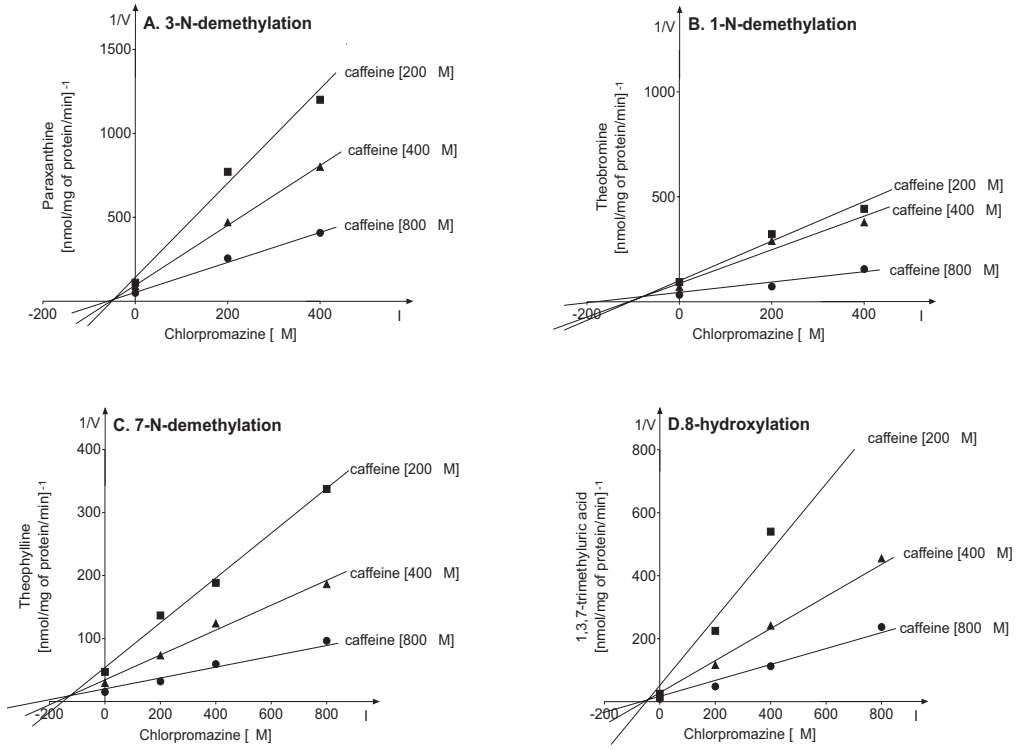

Fig. 4. The influence of chlorpromazine on the metabolism of caffeine in rat liver microsomes (Dixon plots). Kinetics of the inhibition of caffeine 3-N-demethylation (A); 1-N-demethylation (B); 7-N-demethylation (C); 8-hydroxylation (D). V = velocity of the reaction, I = concentration of inhibitor

and possibly other CYP isoenzymes (inhibition of 7-N-demethylation), e.g. CYP2B2 and/or CYP2E1, by the neuroleptics. As reflected by K_i values, the potency of inhibition of caffeine oxidation by perazine was similar to that of levomepromazine (Fig. 2 a, b, c, d; Tab. 1). Thioridazine was a weaker inhibitor of caffeine 3-N- and 7-N-demethylation and 8-hydroxylation than levomepromazine (Fig. 3 a, b, c, d; Tab. 1). Chlorpromazine was weaker than levomepromazine in inhibiting caffeine 1-N-demethylation and 8-hydroxylation, and could hardly inhibit 7-N-demethylation of the marker substance (Fig. 4 a, b, c, d; Tab. 1), The *in vitro* observed interactions of neuroleptics with cytochrome P-450 should be important in *in vivo* conditions since the calculated K_i values were within the presumed concentration range of the inhibitors in the liver *in vivo* (i.e. below 100 μM), both in pharmacological experiments and in psychiatric patients [1, 2, 6, 7, 12, 13, 15].

The investigated phenothiazine neuroleptics exhibit similar inhibitory potencies towards caffeine oxidation (in terms of both metabolic pathway specificity and potency) to earlier studied imipramine with its broad and distinct inhibition of caffeine metabolism. But they differ from other antidepressant drugs, e.g. amitriptyline, a weak CYP3A2 inhibitor or fluoxetine, a weak CYP1A2 inhibitor, as shown by high inhibitory constants for caffeine 8-hydroxylation and 3-N-demethylation, respectively [14].

As mentioned in the Introduction, our earlier studies as well as data of other authors point to species differences in the contribution of cytochrome P-450 isoenzymes to the metabolism of phenothiazine neuroleptics. For example, N-demethylation of perazine in humans was shown to be catalyzed by CYP3A4 and CYP2C9 [23] while in the rat no influence of specific CYP3A and CYP2C inhibitors on perazine N-demethylation was observed. The present work indicates that there are also species differences in the inhibition of caffeine oxidation pathways, i.e. in the inhibition of the isoenzymes CYP1A2 and CYP3A2 by neuroleptics. Our studies carried out on rats showed competitive inhibition of both CYPs by the investigated phenothiazines, while experiments carried out on human liver microsomes revealed that out of four phenothiazine neuroleptics (perphenazine, thioridazine, chlorpromazine, fluphenazine) only perphenazine and fluphenazine exerted moderate inhibition of CYP1A2-catalyzed phenacetin O-deethylation [22]. Hence, this suggests certain structural differences in the catalytic sites of the two mentioned enzymes between humans and rats.

In summary, the obtained results showed that all the investigated neuroleptics had broad spectra of CYP inhibition in the rat liver. The isoenzymes CYP1A2 and CYP3A2 were distinctly inhibited by all the investigated neuroleptics, while other CYP isoenzymes (CYP2B and/or 2E1) by perazine and levomepromazine. The CYP3A2 inhibition was most pronounced. The obtained results require further consideration, since the investigated CYP isoenzymes (CYP1A2 and CYP3A) contribute to the metabolism of endogenous substances, number of drugs and carcinogens. It seems, therefore, advisable to carry out analogical experiments concerning phenothiazines and caffeine oxidation on human liver microsomes.

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