THE *IN VITRO* HEPATIC MICROSOMAL METABOLISM OF 3,5-DIMETHYL-4-(PHENYLAZO)-(1H)-PYRAZOLE IN RATS

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SUMMARY

The *in vitro* hepatic microsomal metabolism of 3,5-dimethyl-4-(phenylazo)-(1H)-pyrazole (DMPAP) was studied using washed rat hepatic microsomal preparations fortified with NADPH. The substrate, DMPAP, and its potential metabolites, i.e. the corresponding reduction product, 3,5-dimethyl-4-amino-(1H)-pyrazole (DMAP), and the oxidation product, 3,5-dimethyl-4-(phenylazoxy)-(1H)-pyrazole (DMAPO), were synthesized and their structures elucidated by use of their spectral characteristics. DMPAP and its potential metabolites were then separated using a reverse phase HPLC system consisting of a C_{18} column and a mobile phase of acetonitrile:water (50:50) at a flow rate of 1 ml/min with UV detection at 254 nm. DMPAP was incubated with rat microsomal preparations, extracted into DCM in the presence of NaCl, and finally evaporated under a stream of nitrogen. The results from HPLC studies showed that DMPAP was metabolised to the corresponding reduction and oxidation products in the presence of NADPH.

KEY WORDS

azopyrazole, microsomes, rat, *in vitro* metabolism

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INTRODUCTION

Azo compounds are widely used as synthetic colourings in food, pharmaceuticals and cosmetics. They are also used as diagnostic and therapeutic agents /1-3/. Azo compounds are metabolised through both oxidation and reduction reactions and consequently their metabolism manifests a complex interplay between these two pathways /1/. The reduction of azo compounds to primary amine is catalyzed by mammalian liver microsomal and cytosolic enzymes, intestinal bacteria and other microorganisms. Metabolic studies of the azo dye prontosil and identification of its reduction products led to the discovery of the first chemotherapeutic agent, sulphanilamide /4/. Depending on the specific compound, azo reduction may result in either detoxification or activation /1,2/. Reduction of carcinogenic azo compounds leads in some cases to loss of carcinogenic activity, while oxidation of azo compounds may promote activation /1,2/. The microsomal processes involved in N-oxidation of azo compounds are thought to be mediated by NADPH-dependent processes, involving either the cytochrome P-450 system or the flavoprotein amine oxidase /5/.

Although the *in vitro* metabolism of bisalkyl, bisaryl and aralkylazo compounds has been previously reported, studies on the *in vitro* metabolism of heterocyclic arylazo compounds are lacking /1-7/. We therefore selected 3,5-dimethyl-4-(phenylazo)-(1H)-pyrazole (DMPAP) (Fig. 1) as a model azo compound in the present study. The aim was to establish whether DMPAP produces an azoxy metabolite or an N-oxidation metabolite on the heterocyclic ring. Another aspect of the study was to investigate whether or not metabolic reduction occurred.

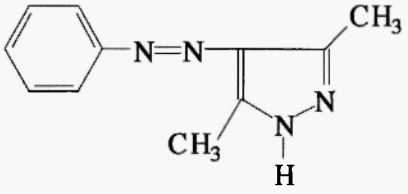

Fig. 1: Structure of DMPAP.

MATERIALS AND METHODS

All chromatographic solvents and chemicals were obtained from Merck. Azo and azoxy compounds were synthesised in our laboratory as described below. The reduction compound 3,5-dimethyl-4-amino-pyrazole (DMAP) was synthesized previously by reduction of DMPAP with hydrazine hydrate without a catalyst and recrystallized just before use (m.p.: 194-195°C) /8/. Melting points were determined on a Buchi 530 melting point apparatus and were uncorrected. Mass spectra of DMPAPO were obtained on a UG Zabspec GC-MS spectrometer.

Synthesis of 3,5-dimethyl-4-(phenylazo)-(1H)-pyrazole (DMPAP)

To synthesize DMPAP, the corresponding diazonium salt was treated with acetyl acetone at 0-5°C and the mixture reacted with hydrazine hydrate. The solid obtained by filtration was recrystallized from ethanol (m.p.: 126-134°C) /8/ (Fig. 2).

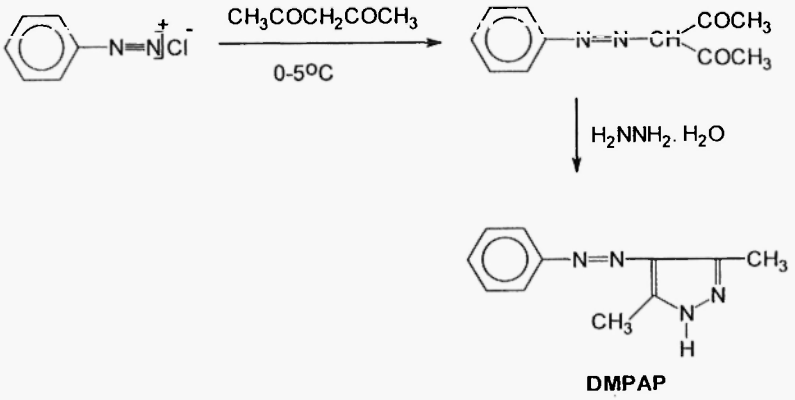

Fig. 2: Synthetic route to DMPAP.

Synthesis of 3,5-dimethyl-4-(phenylazoxy)-(1H)-pyrazole (DMPAPO)

DMPAP was dissolved (2.5 mmol, 0.5 g) in glacial acetic acid and 30% hydrogen peroxide added dropwise at 0-5°C. The reaction mixture was stirred overnight at room temperature and made alkaline with sodium hydroxide. The mixture was treated with diethylether and the solvent removed under reduced pressure. The residue was purified

by silica gel column chromatography with a solvent system of dichloroethane:acetone (80:20, v/v). Evaporation of the solvent gave DHPAPO as a yellow solid, yield 20%, m.p.. 83-85°C /9/ (Fig. 3).

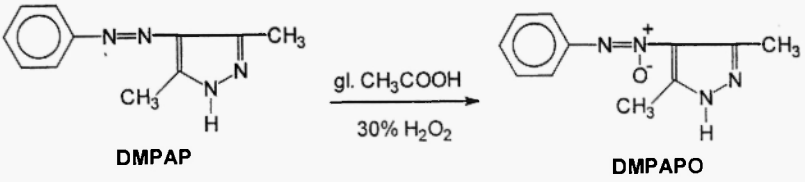

Fig. 3: Synthetic route to DMPAPO.

High performance liquid chromatography

The substrate and its potential metabolites were analysed using a reverse-phase isocratic HPLC system which comprised a model 302 Gilson pump, a model 7125 syringe loading sample injector valve fitted with a 20-µl sample loop, a SpectroMonitor III Model 1204A LDC variable wavelength UV detector and a Tekman recorder. The analytical column (Phase Separations Ltd., Deeside, UK) contained Spherisorb 5-µm ODS (250 x 4.6 mm i.d.) and the pre-column material was co-pellicular ODS (Whatman International Ltd., Maidstone, Kent). The mobile phase composition was acetonitrile:water (50:50, v/v) at a flow rate of 1 ml/min. The substrate and its potential metabolites were detected at 254 nm. HPLC retention times of the substrate DMPAP, its potential metabolites DMPAPO, the reduction compound DMAP and aniline were 9.3, 7.3, 3.3 and 5 min, respectively.

Incubation and extraction procedures

β-Nicotinamide dinucleotide phosphate (disodium salt; NADP) and glucose-6-phosphate (disodium salt; G-6-P) were purchased from BDH. Glucose-6-phosphate dehydrogenase suspension (Reinheit grade II, 10 mg per 2 ml; G-6-PD) was obtained from Boehringer Mannheim Corporation (London). Dichloromethane was obtained from Merck.

Adult male Wistar rats were used in this study. The animals were

deprived of food overnight prior to sacrifice, but were allowed water *ad libitum*. They were previously fed on a balanced diet. Washed hepatic microsomes were prepared as described by Schenkman and Cinti /10/.

Incubations were carried out in a shaking water-bath at 37°C using a standard cofactor solution consisting of NADP (2 μmol), G-6-P (10 μmol), G-6-PD suspension (1 unit) and aqueous $MgCl_2$ (50% w/w) (20 μmol) in phosphate buffer (0.2 M, pH 7.4, 2 ml) at pH 7.4. Cofactors were pre-incubated for 5min to generate NADPH, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (5 μmol) in methanol (5 μl). The incubation was continued for 30 min, terminated and extracted with dichloromethane (2 x 5 ml) in the presence of NaCl. The organic extracts were evaporated to dryness under a stream of nitrogen at 20°C. The residues were reconstituted in 200 μl methanol for HPLC. The reconstituted extracts were analysed using the reverse-phase isocratic HPLC system described above.

RESULTS AND DISCUSSION

The H_2O_2 oxidation product of DMPAP was confirmed as the azoxy compound, DMPAPO, following evaluation of the fragmentation patterns from GC-MS analysis (Fig. 4). It was demonstrated that oxidation occurred on the azo nitrogen next to the heterocyclic ring but not in the heterocyclic ring (Fig. 4).

Following the *in vitro* metabolism of DMPAP, the corresponding oxidation (DMPAPO) and two reduction metabolites (DMAP and aniline) were detected by HPLC. These metabolites showed identical chromatographic behaviour as the authentic compounds. Figure 5 shows an HPLC chromatogram following metabolism of DMPAP by rat microsomes. No metabolites were observed in control experiments either in the presence of denatured microsomes or in the absence of cofactors. This indicates that the reaction requires an enzyme source and NADPH as a cofactor (Fig. 5).

The observations in the present study clearly indicate that aromatic heterocyclic azo compounds can also be converted into azoxy and reductive metabolites by microsomal preparations as for other azo compounds previously studied /1-7/. These two separate processes, therefore, must be considered a general metabolic route for any type of

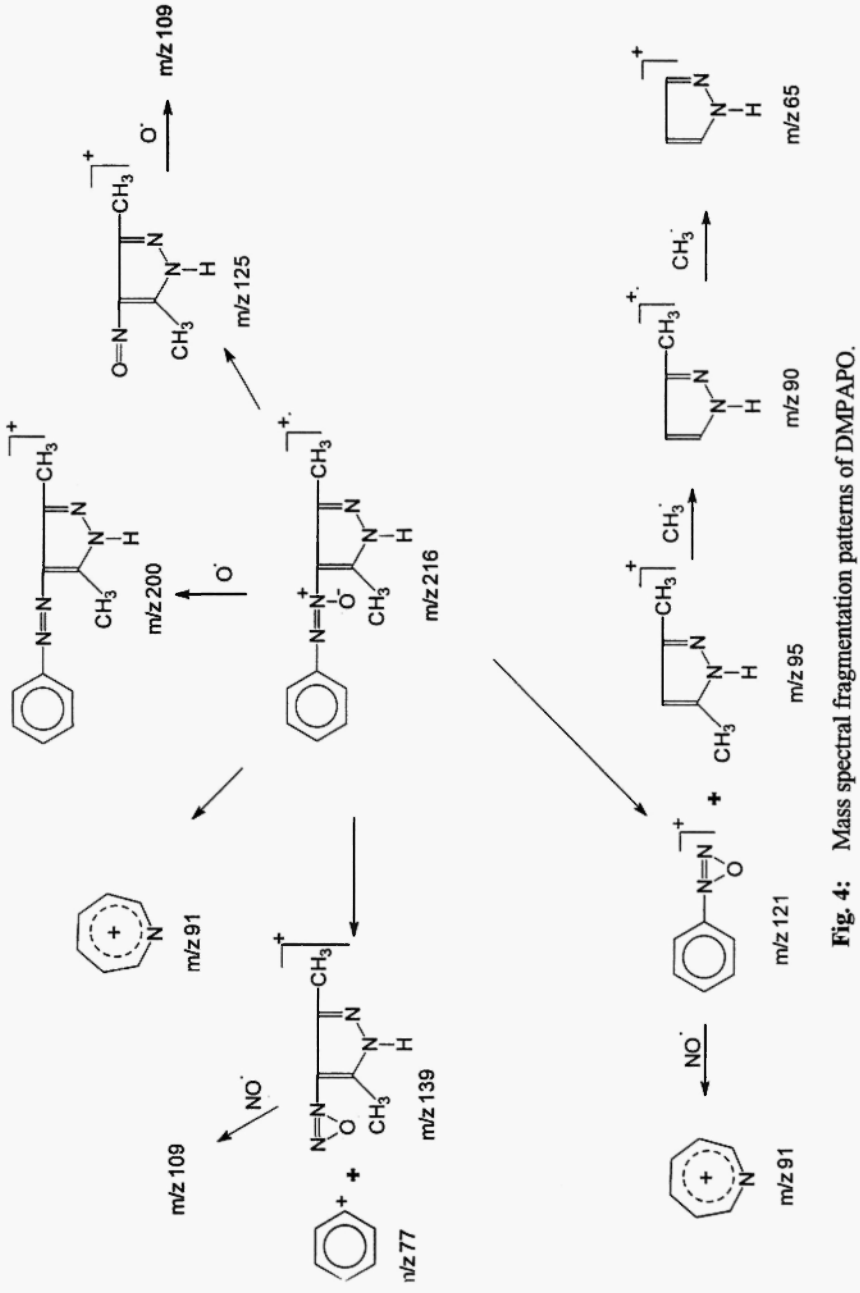

Fig. 4: Mass spectral fragmentation patterns of DMPAPO.

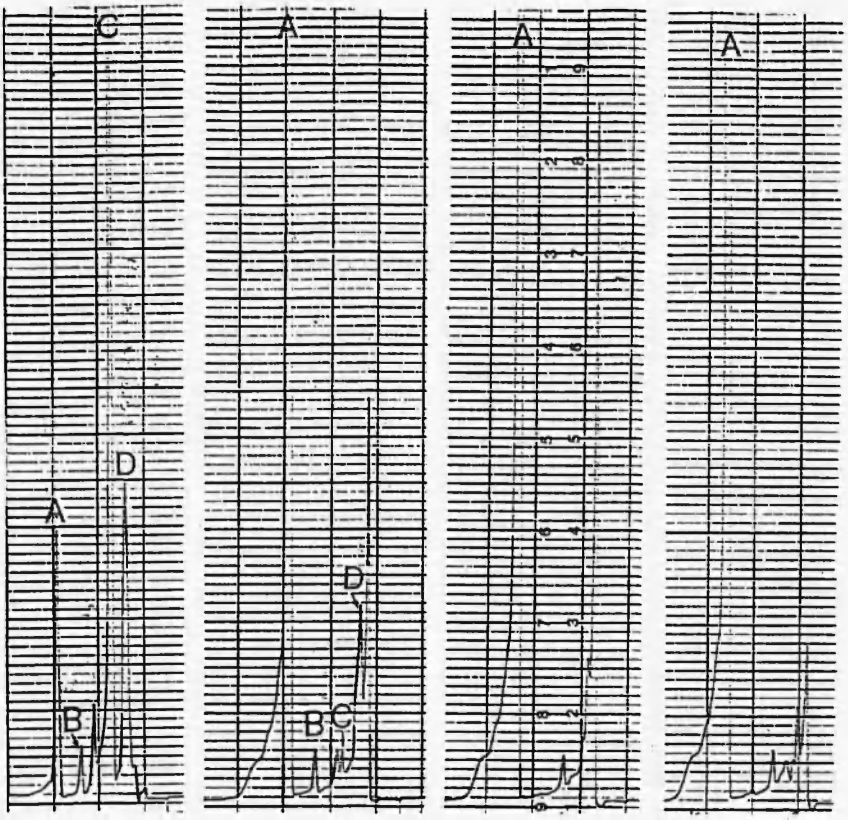

Fig. 5: Reversed phase HPLC chromatogram obtained: A. from authentic DMPAP and its potential metabolites; B. following extraction of male rat microsomal incubation mixture with DMPAP as substrate; C. control with boiled microsomes; D. control without cofactors. (Peaks: A= DMPAP; B= DMPAPO; C= Aniline; D= DMAP.)

azo compound. It was also observed that metabolic N-oxidation occurred in the azo moiety rather than the heterocyclic ring nitrogen. Figure 6 shows the established metabolic products of DMPAP.

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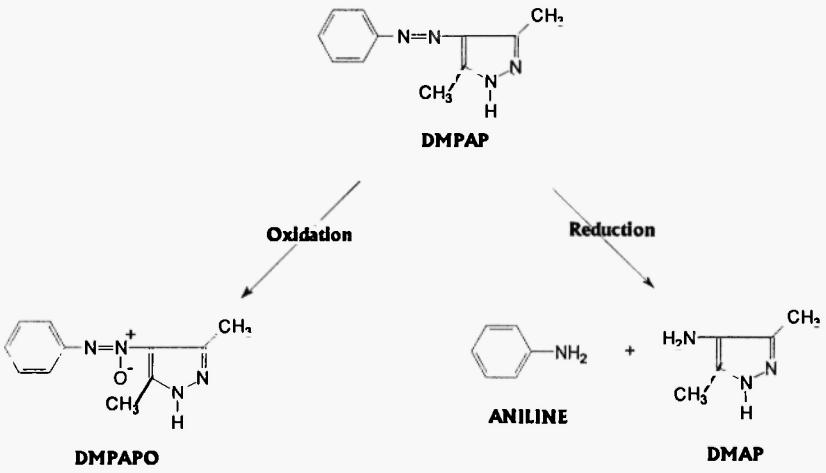

Fig. 6: Established metabolites of DMPAP.

REFERENCES

1. Zbaida S. The mechanism of microsomal azo reduction: Predictions based on electronic aspects of structure-activity relationships. Drug Metab Rev 1995; 27: 497-516.
2. Levine WG. Metabolism of azo dyes: Implication for detoxication and activation. Drug Metab Rev 1991; 23: 253-309.
3. Koh MH, Gorrod JW. In vitro metabolic N-oxidation of azo compounds I. Evidence for formation of azo N-oxides (azoxy compounds). Drug Metab Drug Interact 1989; 7: 253-272.
4. Mitchard M. Bioreduction of organic nitrogen. Xenobiotica 1971; 1: 469-481.
5. Koh MH, Gorrod JW. In vitro metabolic N-oxidation of azo compounds III. Effect of various potential inhibitors, activators and inducers on N-oxidation. J Biopharm Sci 1990; 1: 191-204.
6. Dunn DL, Lubet RA, Prough RA. Oxidative metabolism of N-isopropyl-α-(2-methylhydrazino)-p-toluamide HCl (procarbazine) by rat liver microsomes. Cancer Res 1979; 39: 4555-4563.
7. Wiebkin P, Prough RA. Oxidative metabolism of N-isopropyl-α-(2-methyl-azo)-p-toluamide (azoprocarbazine) by rodent liver microsomes. Cancer Res 1980; 40: 3524-3529.
8. Ergenç N, Rollas S. Some azopyrazoles I. J Pharm Fac lst 1975; 11: 138-157.
9. Richter V. The Chemistry of the Carbon Compounds. Volume III. The Aromatic Compounds. New York: Elsevier, 1946; 133-135.
10. Schenkman JB, Cinti DL. Preparation of microsomes with calcium. Meth Enzymol 1978; 52: 83-85.