CYTOCHROME P-450 AND FAD-MONOOXYGENASE MEDIATED S- AND N-OXYGENATIONS

L.A. Damani* and A.A. Houdi**
**Chelsea Department of Pharmacy*
King's College London, Chelsea Campus
Manresa Road, London, SW3 6LX
and
***VA Medical Center 111C*
Lexington, Kentucky, 40511, U.S.A.

CONTENTS

SUMMARY

S- and N-Oxygenations are mostly mediated by either the cytochrome P-450 group of enzymes (haemoproteins), or the FAD-containing monooxygenases (flavoproteins). The nucleophilicity of the heteroatom may be an important determinant of which of these microsomal enzymes is utilised for specific oxygenations. This article describes the approaches that could be used for assessing the relative contribution of these microsomal oxidases to a particular reaction, with reference to studies with N,N-dimethylaniline, diethylsulphide, tetrahydrothiophen and dibenzothiophen.

I. INTRODUCTION

Metabolic S- and N-oxygenations represent important routes of metabolism for a large number of drugs and other foreign compounds. Therefore, there has been an interest in investigating the enzymic basis for such reactions, particularly where N-oxides or sulphoxides are major metabolites. Any changes in the activity of enzymes involved in specific oxygenations would have an effect on drug disposition, and an effect ultimately on drug pharmacology and/or toxicology. Most S- and N-oxygenations are mediated by either the cytochrome P-450 group of enzymes (haemoproteins), or the FAD-containing monooxygenses (flavoproteins). Data emerging appears to suggest that nucleophilicity may be an important determinant of which of these two microsomal oxidases is utilised for oxygenations at the appropriate heteroatom.

Early studies on metabolic N-oxygenation (N-oxide formation) suggested that N-oxidation of tertiary aliphatic or alicyclic amines, or of N,N-dialkylarylamines, was mediated by the flavin-containing monooxygenase (EC.1.14.13.8) and not the cytochrome P-450 system (see review articles 1-3). Metabolic N-oxidation of the less nucleophilic pyridine, and other aromatic azaheterocycles, is however mediated by a phenobarbitone- inducible cytochrome P-450 system /4,5/; the flavin enzyme appears not to be able to catalyse oxidation at a tertiary heteroaromatic amino functionality /1/.

There are however some inconsistencies in the literature; the N-oxidation of N,N-dmethylaniline has been claimed to involve both cytochrome P-450 and the flavin-monooxygenase /6,7/. N,N-

Dimethylaniline is potentially an excellent model substrate for both the mixed function oxygenases (Figure 1). Indeed we had proposed to use this single compound routinely in our laboratory for monitoring the activity of cytochrome P-450 (mediating N-demethylation) and flavin-monooxygenase (mediating N-oxidation) in rat hepatocytes maintained in monolayer culture /8/. In view of the reports by Hlavica and co-workers /6,7/ claiming involvement of cytochrome P-450 in the N-oxidation of this proposed model substrate, it was essential that we ascertained the relative contributions of the two microsomal monooxygenases to the N-oxidation of N,N-dimethylaniline. In addition, we felt there was a need to look at other substrates as potential probes for the flavin-monooxygenase.

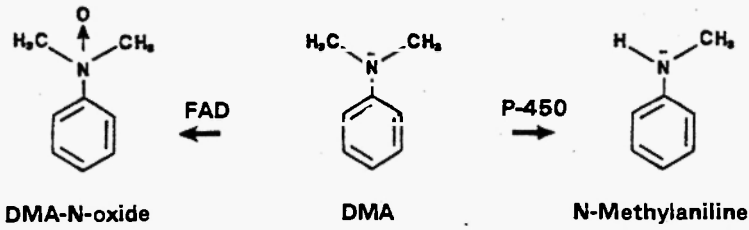

Fig. 1: N,N-Dimethylaniline, a single model substrate for monitoring the activity of both the microsomal mixed function monooxygenases.

Although early work on chlorpromazine had suggested that the principal enzyme involved in sulphoxidation was cytochrome P-450 linked by analogy with metabolic N-oxidation /2,9/, the flavin enzyme would be expected to be a better S-oxygenase for the more nucleophilic divalent sulphur of aliphatic and nonaromatic heterocyclic sulphides. We therefore decided to evaluate some thioethers as potential specific substrates for the flavin enzyme.

The present short review outlines data from studies carried out to delineate the role of the two monooxygenases in the N-demethylation and N-oxidation of N,N-dimethylaniline (DMA), and in the sulph-oxidation of diethylsulphide (DES, an aliphatic sulphide), tetra-hydrothiophen (THTP, a nonaromatic heterocyclic sulphide) and dibenzothiophen (DBTP, an aromatic heterocyclic sulphide). Micro-somes from control, or phenobarbitone (PB) pretreated (80 mg/Kg

i.p. daily, 3 days), or -naphthoflavone (BNF) pretreated (100 mg/Kg i.p. daily, 3 days) male Sprague-Dawley rats were prepared by standard centrifugation techniques /10/. Typical reactions (37°C) were performed in 2 ml Tris/KCl buffer pH 7.4, 5μmole substrate and an NADPH-regenerating system, and were initiated by addition of 6-8 mg microsomal protein. Co-factor concentrations were optimised for each substrate, and product formation was linear with respect to time (10 min) and protein concentration under these conditions. N-Methylaniline and DMA N-oxide were monitored by an HPLC method /11/ the sulphoxides of DES and THTP were measured by GLC, and the sulphoxide of DBTP by an HPLC assay /12,13/.

IL EFFECT OF INDUCERS, ACTIVATORS AND INHIBITORS ON METABOLIC S- AND N-OXYGENATIONS

A very useful approach for establishing the role of a particular enzyme in a metabolic reaction is to use an inducer, activator or a specific inhibitor.

Differences in response to various pretreatments, or to chemicals included in incubation media, were initially utilised in differentiating the enzyme system(s) involved in oxidation reactions. Activities in control microsomes (nmoles/mg protein/min) were as follows: DMA N-demethylation = 2.2; DMA N-oxidation = 1.0; THTP-sulphoxidation = 2.4; DES-sulphoxidation = 2.3, and DBTP-sulphoxidation = 1.4. Phenobarbitone pretreatment increased the activity of DMA N-demethylation and DBTP-sulphoxidation 2.5-fold and 3.0-fold respectively. -Naphthoflavone selectively induced DBTP-sulphoxidation; neither inducer had any inductive effect on DMA N-oxidation, or on THTP- and DES- sulphoxidation. Typical cytochrome P-450 inhibitors (e.g. carbon monoxide, SKF 525A, metyrapone) inhibited DMA N-demethylation and DBTP-sulphoxidation. *n*-Octylamine inhibited N-demethylation of DMA as well as the sulphoxidation of DBTP, whereas it enhanced the N-oxidation of DMA and the sulphoxidation of THTP and DES (about 2-fold in each case). These latter reactions were inhibited by 1-naphthylthiourea. These data suggested that DMA N-demethylation and DBTP-sulphoxidation are cytochrome P-450 reactions, where DMA N-oxidation, and THTP and DES-sulphoxidations are catalysed by

the flavin enzyme. These results were confirmed by use of pure enzyme and differential heat inactivation experiments (see below).

III. DIFFERENTIAL HEAT INACTIVATION OF MICROSOMAL P-450 AND FLAVIN ENZYMES

The FAD-containing monooxygenase is unstable at 37°C in the absence of NADPH, resulting in an irreversible conformational change and loss of enzymic activity /14/. The microsomal P-450 system, on the other hand, is unstable at 37°C in the presence of NADPH /15/. This differential inactivation of microsomal P-450 and flavin enzymes, by heating microsomes at 37°C in the presence and absence of NADPH for 20 minutes, can be used as another approach for determining the nature of enzymes catalysing specific oxidations. Specific activities for DMA N-oxidation (see Table 1), THTP-sulphoxidation and DES-sulphoxidation were not altered in microsomes maintained at 37°C for 20 minutes in the presence of NADPH; DMA N-demethylation (Table 1) and DBTP-sulphoxidation

TABLE 1

N-Oxidation and N-demethylation of N,N-dimethylaniline with rat liver microsomes preincubated at 37°c in the presence and absence of NADPH

Enzymatic activity (a)	Microsomal Treatment (b)		
	No pretreatment	Pretreatment with NADPH	Pretreatment without NADPH
DMA N-oxidation	1.04 ± 0.04 (100)	0.99 ± 0.004 (96)	0.053 ± 0.03 (6)
DMA N-demethylation	2.17 ± 0.1 (100)	0.61 ± 0.05 (28)	2.14 ± 0.09 (99)

(a) = nmole product/min/mg/protein ± S.D., n = 4 or more, percentage of control values in parenthesis.

(b) = microsomes heated at 37°C for 20 minutes in the presence or absence of an NADPH regenerating system

were markedly reduced in these microsomes (28% and 5% of control values respectively). Almost exactly the opposite occurs with microsomes heated at 37°C for 20 minutes in the absence of NADPH; N-oxidation of DMA and sulphoxidation of THTP and DES was drastically reduced (5%, 30% and 28% of control values respectively), but this pretreatment had no effect on the P.450 pathways.

IV. S- AND N-OXYGENATIONS WITH PURIFIED CYTOCHROME P-450 AND FAD-CONTAINING MONOOXYGENASES

Pure hepatic cytochrome P-450 and P-448 isozymes, prepared from PB- and BNF- induced rats, were obtained from Dr. G.G. Gibson (University of Surrey, UK). Pure hog liver FAD-containing monooxygenase was a gift from Professor D.M. Ziegler (University of Texas, Austin, USA). Reconstitution studies demonstrated that DMA N-demethylation was catalysed by P-450 (51.3 ± 0.5 nmole/min/rmole P-450) and P-448 (14.5 ± 0.7 nmole/min/nmole P-448), but N-oxidation of this substrate was not catalysed by either isozyme. The pure hog liver FAD-monooxygenase readily oxidised DMA, and in addition sulphoxidised THTP and DES (85 ± 0.7, 170 ± 1.2 and 240 ± 1.0 nmole/min/mg protein respectively); the flavin enzyme did not N-demethylate DMA, or sulphoxidise DBTP.

V. CONCLUDING REMARKS

On the basis of data presented here, and also earlier published data, it would seem that nucleophilicity is an important determinant of which of of the two monooxygenases is utilised for S- and N-oxygenations. N-Oxygenation of nucleophilic trialkylamines (e.g. trimethylamine) and N,N-dialkyarylamines (e.g. N,N-dimethylaniline) is a flavoprotein reaction /1,3,9,16/ whereas N-oxygenation of the less nucleophilic heterocyclic aromatic amines (e.g. pyridine) is by cytochrome P-450 (see Figure 2). This concept appears to apply equally to S-oxygenation of thioethers; dialkysulphides being substrates for the flavin, and diarylsulphides being substrates for the P-450 enzymes. It is interesting that *p*-tolylethylsulphide, a compound of intermediate nucleophilicity, is oxidised by both enzyme systems

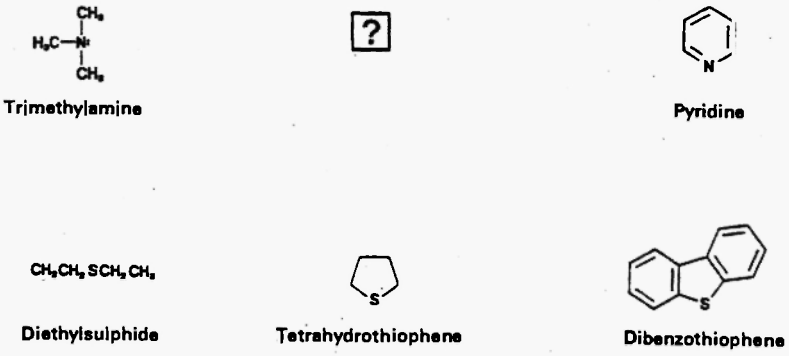

Fig. 2: Cytochrome P-450 and FAD-containing monooxygenase catalysed N- and S- oxygenations in compounds of differing nucleophilicities. Trimethylamine, diethylsulphide and tetrahydrothiophen are substrates for the flavin-containing monooxygenase; pyridine and dibenzothiophene are substrates for cytochrome P-450 enzyme(s). See text for further details.

/17/. Whether any intermediate amines, i.e. alkyldiarylamines, are substrates for both enzyme systems is as yet not known.

It is evident that N-oxidation of DMA and sulphoxidations of THTP and DES in rat microsomes are mediated exclusively via the flavin-monooxygenase. N-Demethylation of DMA and sulphoxidation of DBTP on the other hand are pure P-450 reactions. N,N-Dimethylaniline is therefore potentially an excellent model compound for monitoring the activities of two different monooxygenases; an estimate of N-oxidase activity reflecting the status of the flavin-containing monooxygenase, whereas N-demethylase activity is a measure of the cytochrome P-450 system. Since relatively simple, highly sensitive and specific HPLC assay methods have been developed by us /11/, we recommend the use of this compound in biochemical investigations, not only with purified or crude enzyme preparations, but also with cellular systems (e.g. hepatocyctes, isolated lung cells, intestinal and colonic cells etc.). We have already successfully evaluated the usefulness of this model compound with hepatocyte suspensions and monolayer cultures /8,18/.

In conclusion, where S- and N-oxygenation reactions are quantitatively major routes of metabolism for drugs, it is important that

efforts be directed towards determining the enzymic basis for these reactions. Approaches described in this review could be employed in such investigations, in particular the use of N,N-dimethylaniline as a "marker" model compound to determine whether a specific oxidation is similar to N-demethylation, or N-oxidation of this marker control. Clearly any information on the influence of environmental, physiological or genetic factors on such N- and S-oxygenation reactions would be extremely useful in understanding and explaining inter-individual differences in drug effects and/or drug disposition.

VI. REFERENCES

1. Ziegler, D.M. Microsomal flavin-containing monooxygenase: Oxygenation of nucleophilic nitrogen and sulphur compounds. In: Jakoby, W.B., ed., Enzymatic basis of detoxication, New York: Academic Press, 1980; 201-227.
2. Damani, L.A. Oxidation at nitrogen centres. In: Jakoby, W.B., Bend, J.R. and Caldwell, J. eds. Metabolic basis of detoxication, New York: Academic Press, 1982; 127-149.
3. Ziegler, D.M. Metabolic oxygenation of organic nitrogen and sulphur compounds. In: Mitchell, J.R. and Horning, M.G., eds., Drug Metabolism and drug toxicity, New York: Raven Press, 1984; 33-53.
4. Gorrod, J.W. and Damani, L.A. Some factors involved in the N-oxidation of 3-substituted pyridines by microsomal preparations *in vitro*. *Xenobiotica,* 1979; **9**:209-218.
5. Gorrod, J.W. and Damani, L.A. The effect of various potential inhibitors, activators and inducers on N-oxidation of 3-substituted pyridines *in vitro*. *Xenobiotica,* 1979; **9**:219-226.
6. Hlavica, P. and Kehl, M. Studies on the mechanism of hepatic microsomal N-oxide formation: The role of cytochrome P-450 and mixed-function amine oxidase in the N-oxidation of N,N-dimethylaniline. *Biochem. J.,* 1977; **164**:487-496.
7. Hlavica, P. and Hulsmann, S. Studies on the mechanism of hepatic microsomal N-oxide formation: N-Oxidation of N,N-dimethylaniline by a reconstituted rabbit liver microsomal cytochrome P-448 enzyme system. *Biochem. J.,* 1979; **182**:109-119.
8. Damani, L.A. and Sherratt, A.J. FAD-monooxygenase activity of rat hepatocytes in monolayer culture. *J. Pharm. Pharmacol.,* 1984; **36**(supl.):31P.
9. Ziegler, D.M. Functional groups bearing sulphur. In: Jakoby, W.B., Bend, J.R. and Caldwell, J., eds., Metabolic basis of detoxication, New York: Academic Press, 1982; 171-184.
10. Gorrod, J.W., Temple, D.J., and Beckett, A.H. The metabolism of N-ethyl-N-methylaniline by rabbit liver microsomes: The measurement of metabolites by gas-liquid chromatography. *Xenobiotica,* 1975; **5**:453-464.
11. Damani, L.A., Houdi, A.A., Sherratt, A.J. and Waghela, D. Estimation of FAD-monooxygenase activity in hepatocytes and microsomes using N,N-dimethylaniline. *J. Pharm. Pharmacol.,* 1984; **36**(supl):30P.
12. Houdi, A.A. and Damani, L.A. Cytochrome P-450 and non- cytochrome P-450 sulphoxidatioon. *J. Pharm. Pharmacol.,* 1984; **36**(supl.):62P.
13. Houdi, A.A. Studies on metabolic sulphoxidation of alkyl and aryl thioethers: Role of cytochrome P-450 and FAD-containing monooxygenases. Ph.D. Thesis, University of Manchester, U.K.
14. Kitchell, B., Rauckmann, E. and Rosen, G.M. The effect of temperature on mixed-function amine oxidase intrinsic fluorescence and oxidative activity. *Mol. Pharmacol.,* 1978; **14**:1092-1098.
15. Houdi, A.A. and Damani, L.A. Sulphoxidation of tetrahydrothiophen and its acyclic analogue diethylsulphide. In: Mitchell, S.C. and Waring, R.H., eds., Sulphur in xenobiotics, Birmingham (U.K.): Birmingham University Press, 1983; 131-138.

16. Gorrod, J.W. Differentiation of various types of biological oxidation of nitrogen in organic compounds. *Chem.-Biol. Interact.,* 1973; 7:289-303.
17. Waxman, D.J., Light, D.R. and Walsh, C. Chiral sullphoxidations catalysed by rat liver cytochromes P-450. *Biochemistry,* 1982; 21:2499-2507.
18. Sherratt, A.J. The status of some Phase I drug metabolising enzymes in rat hepatocytes maintained in primary monolayer culture. Ph.D. Thesis, University of Manchester, U.K.