Biochemical Pharmacology, Vol. 30, No. 17, pp. 2451–2456, 1981.
Printed in Great Britain.

0006-2952/81/172451-06 $02.00/0

BIPHASIC *O*-DEETHYLATION OF PHENACETIN AND 7-ETHOXYCOUMARIN BY HUMAN AND RAT LIVER MICROSOMAL FRACTIONS

ALAN R. BOOBIS,* G. CLARE KAHN, CHERRY WHYTE, MARTIN J. BRODIE and DONALD S. DAVIES

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London W12 0HS, U.K.

(*Received* 18 *December* 1980; *accepted* 31 *March* 1981)

Abstract—Human and rat liver microsomal fractions exhibit non-linear Michaelis–Menten kinetics in the *O*-deethylation of both phenacetin and 7-ethoxycoumarin. Comparison of various models indicated that the data were best described by a biphasic plot, which could be interpreted in terms of two populations of cytochrome P-450. The K_m's of the high affinity phase of 7-ethoxycoumarin *O*-deethylase activity were $1.8 \pm 0.4\ \mu M$ and $2.3 \pm 0.4\ \mu M$ for human and rat respectively while the K_m's of the low affinity phase were $205 \pm 20\ \mu M$ and $237 \pm 59\ \mu M$ in the two species respectively. V_{max} of the high affinity phase of human 7-ethoxycoumarin *O*-deethylase activity was 96.9 ± 19.0 pmol mg^{-1} min^{-1} and the activity of the corresponding phase in the rat was 2.7 times greater. The activities of the low affinity phase were 10–15 times greater than the respective activity of the high affinity phase. Rat and human also had similar values for the K_m's of the two phases of phenacetin *O*-deethylase activity, around $5\ \mu M$ for the high affinity phase and $300\ \mu M$ for the low affinity phase. Total activity was very similar in the two species, 1500–1750 pmol mg^{-1} min^{-1} and the difference between the two phases of activity was only 2.5-fold in man and 10-fold in rat.

Studies on the effects of the *in vitro* modifiers of monooxygenase activity α-naphthoflavone and metyrapone further supported the hypothesis that the two phases of *O*-deethylase activity represent two different forms or populations of cytochrome P-450.

The multiplicity of rat liver cytochrome P-450 is well established [1] and there is increasing evidence for a similar multiplicity in man [2]. Studies with purified cytochrome P-450 have shown that the different forms are under separate genetic control [3] and vary in their substrate specificity [4], regiospecificity [5], and in their response to inducers [6] and inhibitors [7]. These properties are the basis of many of the different approaches used in studies of cytochrome P-450 multiplicity. Thus, there are reports on differences in substrate specificity [e.g. 8] and metabolite profiles [e.g. 9] which have been attributed to differences in cytochrome P-450 population. Although the different forms of cytochrome P-450 do vary in substrate specificity, there is considerable overlap in this specificity [1]. Thus, although varying in their affinity for a particular substrate the different forms of the enzyme will still generate the same product, albeit at different rates. When the difference in affinities is large, over ten-fold say, it should be possible to detect the activities of different forms of cytochrome P-450 by kinetic analysis of the formation of a single end product.

Several studies have suggested that for some substrates product formation follows biphasic Michaelis–Menten kinetics, which could be indicative of the involvement of more then one form of cytochrome P-450. Substrates showing biphasic kinetics include amylobarbitone [10], benzo(*a*)pyrene [11], aniline [12], ethylmorphine [13], aminopyrine [14], acetanilide [15] and 7-ethoxycoumarin [16]. In this last example [16] evidence from selective induction studies strongly implicated at least two different forms of cytochrome P-450 in 7-ethoxycoumarin *O*-deethylation.

In our studies on the multiplicity of human cytochrome P-450 it would be of some advantage to use a substrate metabolised by more than one form of cytochrome P-450 with very different affinities for the substrate. We have therefore investigated the kinetics of 7-ethoxycoumarin and phenacetin *O*-deethylation by human and rat liver microsomal fractions.

MATERIALS AND METHODS

Male Wistar rats, 4–6 weeks of age were maintained under constant lighting and temperature cycles. Animals were permitted free access to food (PRD diet, Labsure Animal Diets, Poole, U.K.) until 18 hr prior to death, and to water *ad lib*. Animals were killed by stunning and exsanguination. Hepatic microsomal fractions were prepared as previously described [17] and frozen in aliquots at −80° resuspended in 0.25 M potassium phosphate buffer pH 7.25 containing 30 per cent (v/v) glycerol.

Human liver samples surplus to histological requirement were obtained by wedge biopsy at laparotomy or from renal transplant donors who had met traumatic deaths. Local Research Ethics Committee permission was obtained to use such material

* Author to whom correspondence should be addressed.

in these studies. All biopsies used in the present study were histologically normal or had minor reactive changes only. Microsomal fractions were isolated and stored as previously described [18].

7-Ethoxycoumarin *O*-deethylase activity was assayed by a minor modification of the method of Greenlee and Poland [16]. Reactions were performed under conditions that were linear with respect to protein and time, 10 μg protein for 10 min with rat microsomal fraction and 25 μg protein for 15 min with human microsomal fraction. Substrate was added at 100-fold the final concentration in 10 μl methanol. Final incubation volume was 1 ml. 7-Hydroxycoumarin was measured spectrophotofluorometrically using an Aminco-Bowman spectrophotofluorometer as described [16].

Phenacetin *O*-deethylase activity was determined by a novel assay utilising gas chromatography–mass spectrometry. Final incubation volume was 1 ml containing 75 mM Tris–HCl buffer pH 7.4, 3 mM magnesium chloride, 1.2 mM NADPH and approximately 100 μg microsomal protein. Phenacetin was added at 50-fold the final concentration in 20 μl methanol. The reaction was started by the addition of NADPH. Blanks contained no NADPH. Incubations were for 10 min at 37° in air in a shaking waterbath.

Protein concentration and time of incubation were in the linear range for both rat and human liver. The reaction was terminated by the addition of 0.5 ml of 0.025 M sodium hydroxide, which brought the pH to 12 (the pK_a of paracetamol is about 9.8). d_3-Paracetamol (1 μg in 10 μl methanol) was added as internal standard and excess phenacetin extracted into 15 ml diethyl ether. Both d_0-paracetamol and d_3-paracetamol were stable at pH 12.0 for at least 1 hr. Samples were neutralised within 1 hr with 1 ml of 1 M potassium phosphate buffer, pH 7.0 which gave a final pH of 7.0. Paracetamol was extracted into 15 ml diethyl ether which was transferred to a clean tube and reduced to dryness in a stream of nitrogen. The residue was transferred to a Reactivial (Pierce Chemical Co., Rockford, IL) with 2 × 0.5 ml diethyl ether which was again reduced to dryness under nitrogen. Samples were reconstituted in 30 μl *N*,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and the trimethylsilyl derivatives formed by incubating at 80° for 45 min in a heating block.

Gas chromotography–mass spectrometry was performed using a Finnigan 3200 quadrupole mass spectrometer interfaced via a glass-lined transfer line and all glass jet separator to a Finnigan gas chromatograph combined with a Finnigan 6100 interactive data system. Samples were injected in BSTFA on to a glass column (5 ft × 4 mm) with 3 per cent OV1 on 100/120 mesh Gas Chrom Q as stationary phase. The carrier gas was helium at a flow rate of 30 ml min^{-1} and the oven temperature was 160°. Under these conditions the retention time of paracetamol was 2.2 min. The mass spectrometer was operated in the selected ion monitoring mode and intensities of fragment ions with *m/z* 230 (from d_0-paracetamol) and *m/z* 283 (from d_3-paracetamol) were recorded. These ions represent loss of a methyl group (M-15) from the trimethylsilyl derivatives. Quantitation was by reference to a standard curve constructed from the peak height ratios of known mixtures of d_0- and d_3-paracetamol against paracetamol concentration.

For inhibitor studies, α-naphthoflavone and metyrapone were dissolved in ethanol and 10 μl added to incubations, control incubations receiving solvent alone.

Protein concentration was measured by a modification [18] of the method of Lowry *et al.* [19] with crystalline bovine serum albumin (fraction V) as standard.

MATERIALS

7-Ethoxycoumarin (Gold Label grade), α-naphthoflavone and metyrapone were from Aldrich Chemical Co. Ltd (Gillingham, U.K.), bovine serum albumin fraction V, NADH, NADPH and 7-hydroxycoumarin (grade II) were from Sigma Chemical Co. (Poole, U.K.), Pierce and Warriner (Chester, U.K.) supplied the BSTFA (in sealed 1 g glass ampoules) and the 3 per cent OV1 on 100/120 mesh Gas Chrom Q was from Applied Science Labs Inc (State College, PA). All solvents were of analytical reagent grade. d_3-Paracetamol was a generous gift from Dr D. Baty (Liverpool, U.K.).

ANALYSIS OF RESULTS

K_m and V_{max} values were obtained initially by graphical analysis of Eadie-Hofstee plots [16]. The values thus obtained were used as first estimates in an iterative programme based on non-linear least squares regression analysis (Biomedical Computer Program, Health Science Computing Facility, Dept. of Biomathematics, School of Medicine, University of California, LA, 1975) to calculate values of K_m and V_{max} to fit the equation

$$v_\tau = \frac{V_{max1} \times S}{K_{m1} + S} + \frac{V_{max2} \times S}{K_{m2} + S}. \quad (1)$$

The asymptotic S.D. of the four parameters thus estimated rarely exceeded 25 per cent.

RESULTS

Both 7-ethoxycoumarin and phenacetin were *O*-deethylated biphasically by rat and human fractions. A typical Eadie-Hofstee plot for phenacetin is shown in Fig. 1 and for 7-ethoxycoumarin in Fig. 2. The four parameters K_{m1}, V_{max1}, K_{m2} and V_{max2} were determined graphically from each plot and the line best fitting equation (1) calculated by non-linear regression in each case. Plots similar to those shown in Fig. 2 were generated. The minimum error mean square for this plot was 1110 and compares with a minimum error mean square of 7080 when these data were fitted to traditional Michaelis–Menten kinetics. When v (observed) was plotted against v (calculated) for the two analyses (Fig. 3) the biphasic plot gave the better fit. The residuals from the biphasic plot were evenly distributed about zero across the whole concentration range whereas the residuals from the monophasic plot exhibited a U-shaped distribution across the concentration range. The biphasic plot is thus a better fit to the data.

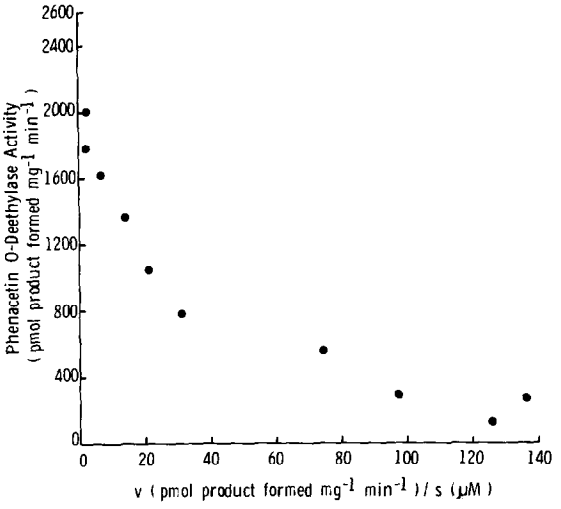

Fig. 1. Eadie–Hofstee plot for phenacetin *O*-deethylase activity of human hepatic microsomal fraction.

Values for the four Michaelis–Menten parameters for 7-ethoxycoumarin *O*-deethylation are shown in Table 1. K_m values for human and rat were very similar, 1.8 ± 0.4 μM and 2.3 ± 0.4 μM respectively for the high affinity components and 205 ± 20 μM and 237 ± 59 μM respectively for the low affinity components. V_{max} values differed considerably between the two species, activity with rat being much greater for both components. V_{max1} for the human was 96.9 ± 19.0 pmol mg^{-1} min^{-1} and for the rat 266 ± 31 pmol mg^{-1} min^{-1}. V_{max2} values were 950 ± 210 pmol mg^{-1} min^{-1} and 3540 ± 830 pmol mg^{-1} min^{-1} for human and rat respectively.

Table 1 also shows values for the four Michaelis–Menten parameters of phenacetin *O*-deethylation. Again, human and rat have similar K_m values but different V_{max} values for the two components of activity. V_{max1} was 440 ± 162 pmol mg^{-1} min^{-1} and 145 ± 55 pmol mg^{-1} min^{-1} in human and rat respectively. The corresponding values for V_{max2} were 1060 ± 250 pmol mg^{-1} min^{-1} and 1600 ± 90 pmol mg^{-1} min^{-1} respectively.

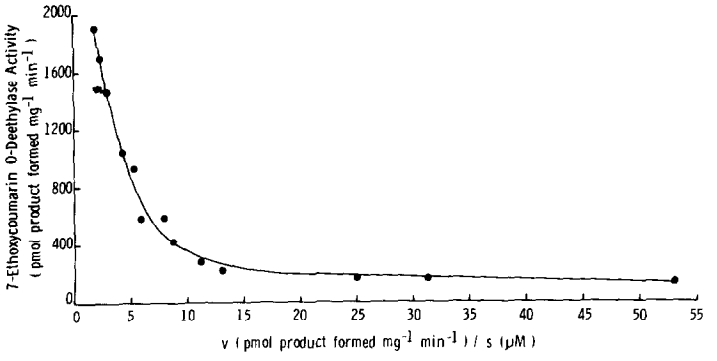

Fig. 2. Eadie–Hofstee plot for 7-ethoxycoumarin *O*-deethylase activity of human hepatic microsomal fraction. Points are experimentally determined values. The solid line is the computer-generated best fit curve as described in the text.

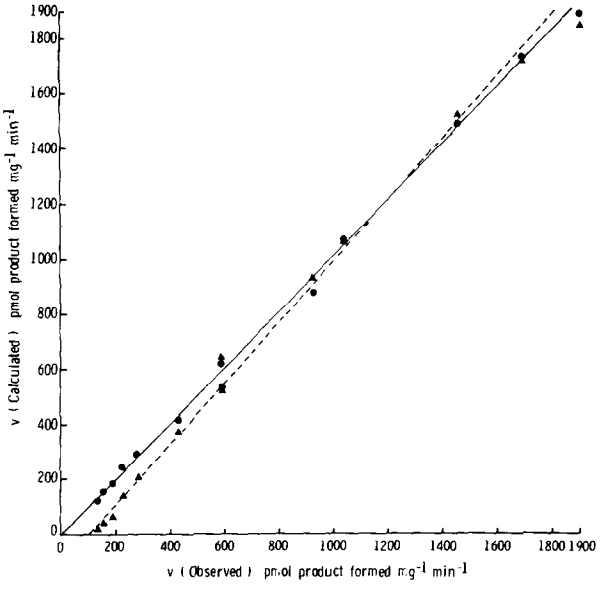

Fig. 3. Plot of v (observed) against v (calculated) for the data shown in Fig. 2. The data were analysed assuming biphasic kinetics (●) and monophasic kinetics (▲).

Table 1. Values for the four Michaelis–Menten parameters of hepatic microsomal 7-ethoxycoumarin *O*-deethylase and phenacetin *O*-deethylase activities

Species	*n*	$V_{max\,1}$ pmol mg^{-1} min^{-1}	K_{m1} μM	$V_{max\,2}$ pmol mg^{-1} min^{-1}	K_{m2} μM
7-Ethoxycoumarin *O*-deethylase activity					
Human	10	96.9 ± 19.0	1.8 ± 0.4	950 ± 210	205 ± 20
Rat	5	266 ± 31	2.3 ± 0.4	3540 ± 830	237 ± 59
Phenacetin *O*-deethylase activity					
Human	5	440 ± 162	6.3 ± 1.5	1060 ± 250	248 ± 75
Rat	3	145 ± 55	4.9 ± 1.8	1600 ± 90	455 ± 28

Values are mean ±S.E.M. for number of samples shown under *n*.

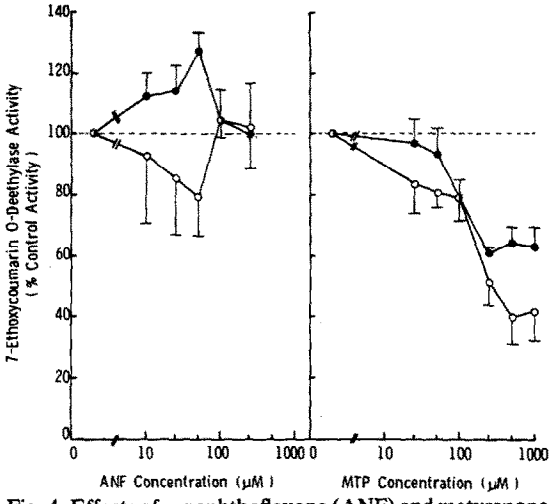

Fig. 4. Effects of α-naphthoflavone (ANF) and metyrapone (MTP) on the two phases of rat hepatic 7-ethoxycoumarin *O*-deethylase activity. High affinity phase (○) and low affinity phase (●). Values are mean ±S.E.M. of 4 animals. Control activity was determined in the presence of vehicle alone.

The possible selective effects of *in vitro* modifiers of monooxygenase activity on the two phases of 7-ethoxycoumarin *O*-deethylation were investigated. Incubations were performed at 7.5 and 1000 μM substrate concentration as representative of the high affinity component and total activity respectively. The activity of the low affinity component was determined by subtraction. With rat liver, concentrations of metyrapone above 250 μM caused greater inhibition of the high affinity component than of the low affinity component of activity while α-naphthoflavone had no significant effect on the high affinity component of activity (Fig. 4). Activity of the low affinity component was enhanced by α-naphthoflavone. With human liver samples metyrapone had effects similar to those in the rat (Fig. 5). The high affinity component was inhibited to a greater extent compared with the low affinity component, at concentrations of metyrapone above 250 μM. Unlike the rat, however, low concentrations of α-naphthoflavone had a greater inhibitory effect than metyrapone on the high affinity component. But, like the rat, the low affinity component of

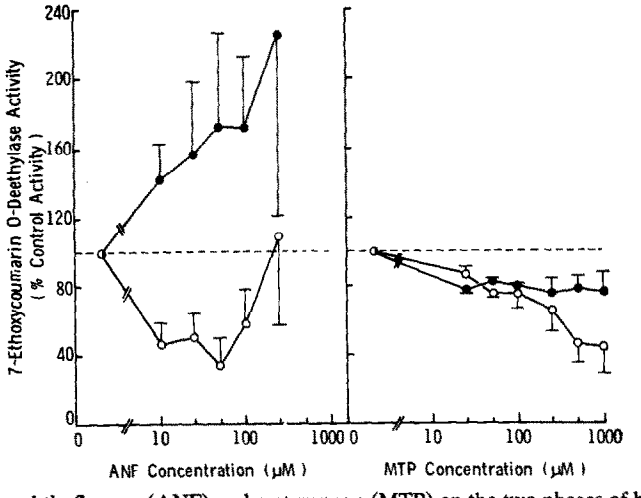

Fig. 5. Effects of α-naphthoflavone (ANF) and metyrapone (MTP) on the two phases of human hepatic 7-ethoxycoumarin *O*-deethylase activity. High affinity phase (○) and low affinity phase (●). Values are mean ±S.E.M. of 3 samples. Control activity was determined in the presence of vehicle alone.

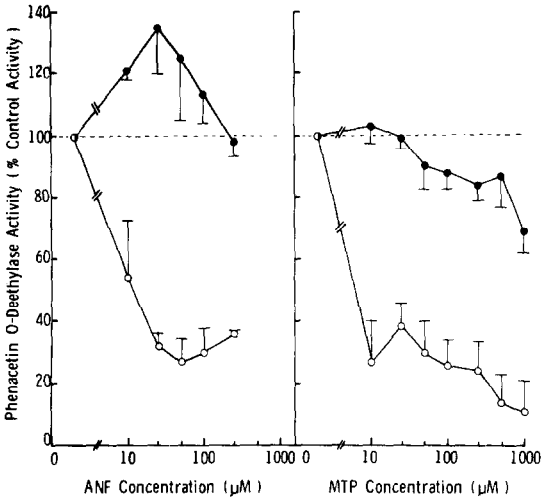

Fig. 6. Effect of α-naphthoflavone (ANF) and metyrapone (MTP) on the two phases of rat hepatic phenacetin O-deethylase activity. High affinity phase (○) and low affinity phase (●). Values are mean ±S.E.M. of 3 animals. Control activity was determined in the presence of vehicle alone.

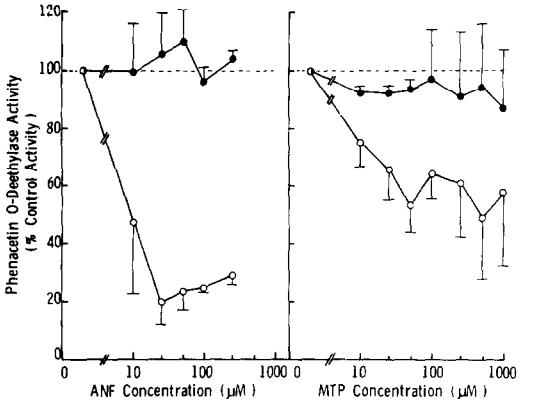

Fig. 7. Effect of α-naphthoflavone (ANF) and metyrapone (MTP) on the two phases of human hepatic phenacetin O-deethylase activity. High affinity phase (○) and low affinity phase (●). Values are mean ± half-range of 2 samples. Control activity was determined in the presence of vehicle alone.

human 7-ethoxycoumarin O-deethylase activity was enhanced by α-naphthoflavone.

The effects of metyrapone and α-naphthoflavone on the two components of phenacetin O-deethylase activity are shown in Fig. 6 for rat and in Fig. 7 for human. Incubations were performed at 20 μM and 2.5 mM and the activity of the two phases calculated as described for 7-ethoxycoumarin. Rat and human liver microsomal fractions gave very similar results. The high affinity component of activity was inhibited more by α-naphthoflavone than by metyrapone at 25 μM, but at higher concentrations metyrapone was more potent than α-naphthoflavone in rat but not in man. The low affinity component was less sensitive to the inhibitory effects of metyrapone than the high affinity component. α-Naphthoflavone had some enhancing effect on the low affinity component of activity in both species.

DISCUSSION

Rat and human liver microsomal fractions O-deethylate both phenacetin and 7-ethoxycoumarin biphasically. This pattern for 7-ethoxycoumarin O-deethylation is similar to that previously reported for mouse [16] and rat [20, 21]. There are a number of possible explanations for biphasic enzyme kinetics. These include solubility effects, diffusion barriers, more than one active site on the enzyme and more than one enzyme. The physicochemical explanations seem unlikely in view of the results with selective modifiers of monooxygenase activity. Guengerich [21] has recently shown that a homogenous preparation of rat cytochrome P-450 O-deethylates 7-ethoxycoumarin monophasically whereas intact microsomal fractions or heterogeneous cytochrome P-450 preparations produce 7-hydroxycoumarin biphasically. Thus, the probable explanation is that the two phases of activity represent the contribution of different populations of cytochrome P-450, one characterised by high affinity and low capacity, the other by low affinity and high capacity. Whether the two populations of cytochrome P-450 involved in phenacetin metabolism are exactly the same as the two populations involved in 7-ethoxycoumarin O-deethylation remains to be determined.

Whereas rat liver is ten-fold more active than human liver in O-deethylating 7-ethoxycoumarin it has similar activity in phenacetin O-deethylation. This provides some slight evidence for the populations of cytochrome P-450 in phenacetin and 7-ethoxycoumarin metabolism being different from each other. Further evidence for this contention comes from the inhibitor studies. Metyrapone is a much more potent inhibitor of the high affinity component of phenacetin O-deethylation at low concentrations, than of the corresponding phase of 7-ethoxycoumarin metabolism. It seems probable that the different populations of cytochrome P-450 involved in the metabolism of the two substrates overlap to some extent, but that there are sufficient differences to produce individual patterns of metabolism and responses to inhibitors.

In view of the recently reported polymorphism in man of debrisoquine, guanoxan, phenacetin [22] and sparteine [23, 24] cytochrome P-450 dependent oxidation, the present results pose intriguing possibilities. Poor metabolisers of phenacetin are still capable of producing quite large amounts of the product, paracetamol [23]. It may be that this phenotype is missing a form of cytochrome P-450 responsible for one phase of phenacetin O-deethylation but at the concentrations of phenacetin occurring *in vivo* the other component of activity is sufficient to produce reasonable amounts of paracetamol. Recent work in this laboratory suggests that this may be so [25].

Acknowledgements—This work was supported by a grant from the MRC. We wish to thank Ms E. A. Morgan for her expert secretarial assistance.

REFERENCES

1. D. E. Ryan, P. E. Thomas, D. Korzeniowski and W. Levin, *J. biol. Chem.* **254**, 1365 (1979).
2. A. R. Boobis, M. J. Brodie, G. C. Kahn and D. S. Davies, in *Microsomes Drug Oxidations and Chemical Carcinogenesis* (Eds. M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette and P. J. O'Brien), Vol. II, p. 957. Academic Press, New York (1980).
3. D. W. Nebert, J. K. Heidema, H. W. Strobel and M. J. Coon, *J. biol. Chem.* **248**, 7631 (1973).
4. F. P. Guengerich, *J. biol. Chem.* **252**, 3970 (1977).
5. F. J. Wiebel, J. K. Selkirk, H. V. Gelboin, D. A. Haugen, T. A. van der Hoeven and M. J. Coon, *Proc. natn. Acad. Sci., U.S.A.* **72**, 3917 (1975).
6. F. Comai and J. L. Gaylor, *J. biol. Chem.* **248**, 4947 (1973).
7. R. L. Norman, E. F. Johnson and U. Muller-Eberhard, *J. biol. Chem.* **253**, 8640 (1977).
8. F. J. Wiebel, H. L. Waters, S. Elliot and H. V. Gelboin, *Biochem. Pharmac.* **25**, 1431 (1976).
9. A. Kramer, H. J. Staudinger and V. Ullrich, *Chem.–Biol. Interact.* **8**, 11 (1974).
10. H. S. Fraser, PhD Thesis, University of London, London (1976).
11. O. Pelkonen, *Res. Commun. chem. Path. Pharmac.* **10**, 293 (1975).
12. F. Wada, H. Shimakawa, M. Tagasugi, T. Kotake and Y. Sakamoto, *J. Biochem.* **64**, 109 (1968).
13. J. R. Hayes, M. U. K. Mgbodile and T. C. Campbell, *Biochem. Pharmac.* **22**, 1517 (1973).
14. T. C. Pederson and S. D. Aust, *Biochem. Pharmac.* **19**, 2221 (1970).
15. T. M. Guenthner, M. Negishi and D. W. Nebert, *Analyt. Biochem.* **96**, 201 (1979).
16. W. F. Greenlee and A. Poland, *J. Pharmac. exp. Ther.* **205**, 596 (1978).
17. S. A. Atlas, A. R. Boobis, J. S. Felton, S. S. Thorgeirsson and D. W. Nebert, *J. biol. Chem.* **252**, 4712 (1977).
18. A. R. Boobis, M. J. Brodie, G. C. Kahn, D. R. Fletcher, J. H. Saunders and D. S. Davies, *Br. J. clin. Pharmac.* **9**, 11 (1980).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. V. Ullrich and P. Weber, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1171 (1972).
21. F. P. Guengerich, *J. biol. Chem.* **253**, 7931 (1978).
22. T. P. Sloan, A. Mahgoub, R. Lancaster, J. R. Idle and R. L. Smith, *Br. med. J.* **2**, 655 (1978).
23. T. Inaba, S. V. Otton and W. Kalow, *Clin. Pharmac. Ther.* **27**, 547 (1980).
24. L. Bertilsson, H. J. Dengler, M. Eichelbaum and H.-U. Schulz, *Eur. J. clin. Pharmac.* **17**, 153 (1980).
25. D. S. Davies, G. C. Kahn, S. Murray, M. J. Brodie and A. R. Boobis, *Br. J. clin. Pharmac.* **11**, 89–91 (1981).