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HIGH MAGNITUDE HEPATIC CYTOCHROME P-450 INDUCTION BY AN N-SUBSTITUTED IMIDAZOLE ANTIMYCOTIC, CLOTRIMAZOLE

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Abstract—A 4-fold induction of hepatic microsomal cytochrome P-450 following 3 days of treatment of rats with clotrimazole (75 mg/kg), a potent monooxygenase inhibitor, greatly exceeded that evident from similar phenobarbital and dexamethasone treatment. The clotrimazole-induced microsomes exhibited a pattern of monooxygenase activities similar to that seen in microsomes from both phenobarbital- and dexamethasone-treated animals. Precautions were necessary to determine both monooxygenase activities and the full amount of cytochrome P-450 present in microsomes because of interference by residual clotrimazole in the microsomes.

In xenobiotic metabolism, imidazoles, especially the N-substituted derivatives, are best known for their inhibition of cytochrome P-450 catalyzed oxidation reactions [1, 2]. Recent interest has centered around the compounds used therapeutically for their antimycotic activity, particularly ketoconazole [3–6]. These compounds also inhibit the cytochrome P-450 dependent reactions involved in mammalian steroid metabolism [7, 8].

Although induction of cytochrome P-450 by N-substituted imidazoles has been reported [6, 9, 10], nothing remarkable relative to the extent was noted. The 2-fold induction by both clotrimazole [6] and *N*-phenylimidazole [10] was characterized as phenobarbital-like by the investigators. Until now, the high magnitude induction reported here for clotrimazole has only been associated with certain polyhalogenated biphenyl derivatives [11, 12] and with nitrogenous compounds such as SKF 525-A [13] and troleandomycin [14] which are capable of forming cytochrome P-450 metabolic-intermediate complexes *in vivo*. For these latter inducers, the complex must be broken by chemical oxidation *in vitro* to determine the full activity of the induced cytochrome. This report describes high magnitude induction with clotrimazole, a nitrogenous compound which does not form a spectrally detectable metabolic-intermediate complex. The induced microsomes had substrate selectivities characteristic of both phenobarbital- and dexamethasone-induced cytochrome P-450 isozymes [15–18], but the degree of cytochrome P-450 induction far exceeded that seen with either.

MATERIALS AND METHODS

Clotrimazole, dexamethasone, dimethylnitrosamine, and the biochemicals were obtained from the Sigma Chemical Co. Ethoxyresorufin and pentoxyresorufin were purchased from the Pierce Chemical Co. and Molecular Probes Inc. respectively. Erythromycin was a gift from Abbot Laboratories, troleandomycin from Pfizer Inc., and SKF 8742-A from Smith Kline & French Laboratories.

Following treatment (phenobarbital, i.p.; dexamethasone in 2% Tween 80, i.p.; and clotrimazole in 30% polyethylene glycol 400, intragastrically) adult male Sprague–Dawley rats were decapitated, and liver microsomal fractions were prepared using established conditions [19]. Protein concentrations were determined by the method of Lowry *et al.* [20]. Enzyme activities were determined after storage of the microsomal fractions for 16–40 hr at −20°.

The microsomal cytochrome P-450 concentration, metyrapone binding, and extent of metabolic-intermediate complex formation from troleandomycin, SKF 8742-A and norbenzphetamine were quantitated using extinction coefficients of 91 mM^{-1} cm^{-1} [21], 68.5 mM^{-1} cm^{-1} [22] and 65 mM^{-1} cm^{-1} [23] respectively. Ethylmorphine, dimethylnitrosamine and erythromycin demethylations were determined from the formaldehyde produced [24]. *p*-Nitroanisole demethylase was determined from the *p*-nitrophenol produced [25], and pentoxy- and ethoxyresorufin dealkylations were determined from the resorufin produced [18, 26]. Aniline hydroxylase activity was determined using a modified method of Imai and Sato [27], and testosterone hydroxylations were determined by a modification of the method described by Waxman *et al.* [28]. Cytochrome P-450 carbon monoxide adduct formation after 30 sec (arbitrary time point) was determined in the dual wavelength mode (450 vs 490 nm) following diothionite addition (2 mg) to a 1.5 ml suspension of microsomes (1 mg protein/ml) that had been gassed extensively (5 min) with CO. Microsomal NADPH–cytochrome *c* (P-450) reductase activity was determined by the method of Masters *et al.* [29].

RESULTS

Clotrimazole, an N-substituted imidazole, is one

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of a select group of agents that can be classified as high magnitude inducers. The magnitude of this induction (4× control) is shown in Table 1. It far exceeded that found for similar doses of phenobarbital and dexamethasone (2.5× control) over a similar time period. In parallel with the higher concentration of cytochrome P-450, the extent of metyrapone binding and of metabolic-intermediate complex formation from SKF 8742-A were also induced much above that seen with either phenobarbital or dexamethasone. Although increases in cytochrome P-450 concentration were much larger, the changes in oxidative metabolism of xenobiotics were often the same as seen with the two established inducers (i.e. not proportional to the large cytochrome changes). After clotrimazole induction, the activity was the same as after dexamethasone for three variables which dexamethasone induced to a greater extent than phenobarbital (ethylmorphine and erythromycin demethylations and metabolic-intermediate complex formation from troleandomycin) and the same as after phenobarbital for two variables which phenobarbital induced more than dexamethasone (pentoxyresorufin and ethoxyresorufin dealkylations). For a variable that both phenobarbital and dexamethasone induced (aniline hydroxylation), and for another which neither phenobarbital nor dexamethasone caused significant induction of (dimethylnitrosamine demethylation), clotrimiazole did not cause any changes. All three agents induced metabolic-intermediate complex formation from norbenzphetamine to the same extent. Demethylation of *p*-nitroanisole was induced most by phenobarbital, intermediate by clotrimazole, and least by dexamethasone. The same rank order was seen for 16β-hydroxylation of, and androstenedione formation from, the endogenous substrate, testosterone (Table 2). For those oxidations induced more by dexamethasone than by phenobarbital (2β-, 6β- and 15β-hydroxylations), clotrimazole-induced microsomes again showed intermediate activity but were generally closer to the activity in phenobarbital-induced microsomes. Of three testosterone hydroxylations induced similarly by dexamethasone and phenobarbital (6α, 7α and 15α), two were induced to a similar extent by clotrimazole, and one (7α) was not affected. Two hydroxylation reactions decreased by dexamethasone (2α and 16α) were also decreased by clotrimazole. The microsomal flavoprotein, NADPH–cytochrome *c* reductase, was induced by clotrimazole, but not to the same extent as by either phenobarbital or dexamethasone (Table 1).

One possible explanation for the monooxygenase activity after clotrimazole never increasing above that seen after phenobarbital or dexamethasone induction is that residual clotrimazole is present in the microsomes and this prevents full expression of activity. Clotrimazole, like many nitrogenous compounds, binds to the heme of cytochrome P-450 [6], and this binding can be monitored as a Type II difference spectrum (Fig. 1). This binding to heme prevents its participation in oxidations [1–4, 6]; the degree of inhibition was approximately proportional to saturation of the heme (Fig. 1). The concentrations giving 50% saturation and 50% inhibition of *p*-nitroanisole demethylation were 0.91 and 0.93 μM for phenobarbital-induced microsomes and 2.02 and 2.20 μM for clotrimazole-induced microsomes. The difference between the values for the two types of microsomes was proportional to the difference in the cytochrome P-450 concentration. In addition to inhibiting monooxygenase activity,

Table 1. Comparison of the influence of the N-substituted antimycotic, clotrimazole, on male rat hepatic drug-metabolizing enzymes with the effects of phenobarbital and dexamethasone

Variable	Control	Phenobarbital	Clotrimazole	Dexamethasone
Treatment (mg/kg × days)*		80 × 4	75 × 3	100 × 3
No. of animals†	10	5	6	5
Cytochrome P-450‡				
Carbon monoxide	0.92 ± 0.04	2.34 ± 0.11	3.73 ± 0.26	2.24 ± 0.07
Metyrapone	0.17 ± 0.02	0.84 ± 0.05	3.14 ± 0.20	1.14 ± 0.19
MI complex‡				
Norbenzphetamine	0.29 ± 0.01 (3)	1.19 ± 0.07 (3)	1.34 ± 0.13 (3)	1.09 ± 0.01 (3)
SKF 8742-A	0.31 ± 0.05 (4)	0.88 ± 0.07 (4)	1.46 ± 0.06 (3)	1.06 ± 0.08 (3)
Troleandomycin	0.07 ± 0.01 (5)	0.31 ± 0.05	1.16 ± 0.08 (3)	1.01 ± 0.21
Dealkylation§				
p-Nitroanisole	0.59 ± 0.05	4.84 ± 0.08 (3)	2.47 ± 0.15	1.73 ± 0.06
Pentoxyresorufin	0.00 ± 0.00 (5)	0.93 ± 0.10 (3)	0.70 (2)	0.00 ± 0.00 (3)
Ethylmorphine	6.06 ± 0.43	14.9 ± 0.2 (3)	23.9 ± 1.5	23.9 ± 1.3 (3)
Erythromycin	0.49 ± 0.04	2.55 ± 0.38	6.52 ± 0.29	6.04 ± 0.38
Dimethylnitrosamine	1.26 ± 0.13	2.01 ± 0.18 (4)	1.41 ± 0.34	1.21 ± 0.28 (3)
Ethoxyresorufin	0.001 ± 0.001 (4)	0.018 ± 0.003 (3)	0.018 ± 0.004 (3)	0.004 ± 0.004 (3)
Hydroxylations§				
Aniline	0.76 ± 0.06	2.07 ± 0.23	0.85 ± 0.14 (4)	1.65 ± 0.18
NADPH–cyt. *c* reductase§	120 ± 7	232 ± 10 (3)	171 ± 11	205 ± 13

* Microsomes were prepared 24 hr after the last dose of phenobarbital and dexamethasone, and 48 hr after the last dose of clotrimazole.

† Not less than this number of microsomal samples were analyzed, unless indicated by parentheses.

§ Expressed in nmol/mg/min (mean ± SEM).

Table 2. Comparison of the influence of the N-substituted antimycotic, clotrimazole, on male rat hepatic testosterone metabolism with the effects of phenobarbital and dexamethasone

Variable	Control	Phenobarbital	Clotrimazole	Dexamethasone
Treatment (mg/kg × days)*		80 × 4	75 × 3	100 × 3
No. of animals	11	9	3	8
Cytochrome P-450†	1.05 ± 0.02	1.84 ± 0.32	3.28 ± 0.19	1.79 ± 0.15
Oxidation‡ at: 2α	1.51 ± 0.17	1.25 ± 0.16	0.37 ± 0.03	0.80 ± 0.09
2β	0.34 ± 0.05	1.65 ± 0.21	2.02 ± 0.09	4.04 ± 0.63
6α	0.05 ± 0.01	0.14 ± 0.03	0.16 ± 0.01	0.17 ± 0.03
6β	1.91 ± 0.12	8.67 ± 1.01	9.69 ± 0.36	14.10 ± 0.63
7α	0.70 ± 0.01	0.26 ± 0.04	0.11 ± 0.01	0.23 ± 0.05
15α	0.10 ± 0.01	0.19 ± 0.02	0.20 ± 0.01	0.29 ± 0.03
15β	0.11 ± 0.01	0.49 ± 0.07	0.81 ± 0.01	1.89 ± 0.29
16α	2.45 ± 0.27	3.31 ± 0.46	1.81 ± 0.10	1.42 ± 0.10
16β	0.23 ± 0.02	2.44 ± 0.41	1.58 ± 0.13	0.72 ± 0.20
Formation‡ of: androstenedione	1.43 ± 0.16	4.07 ± 0.61	3.39 ± 0.12	2.93 ± 0.37

* Microsomes were prepared 24 hr after the last dose of phenobarbital and dexamethasone, and 48 hr after the last dose of clotrimazole.

† Expressed in nmol/mg (mean ± SEM).

‡ Expressed in nmol/mg/min (mean ± SEM).

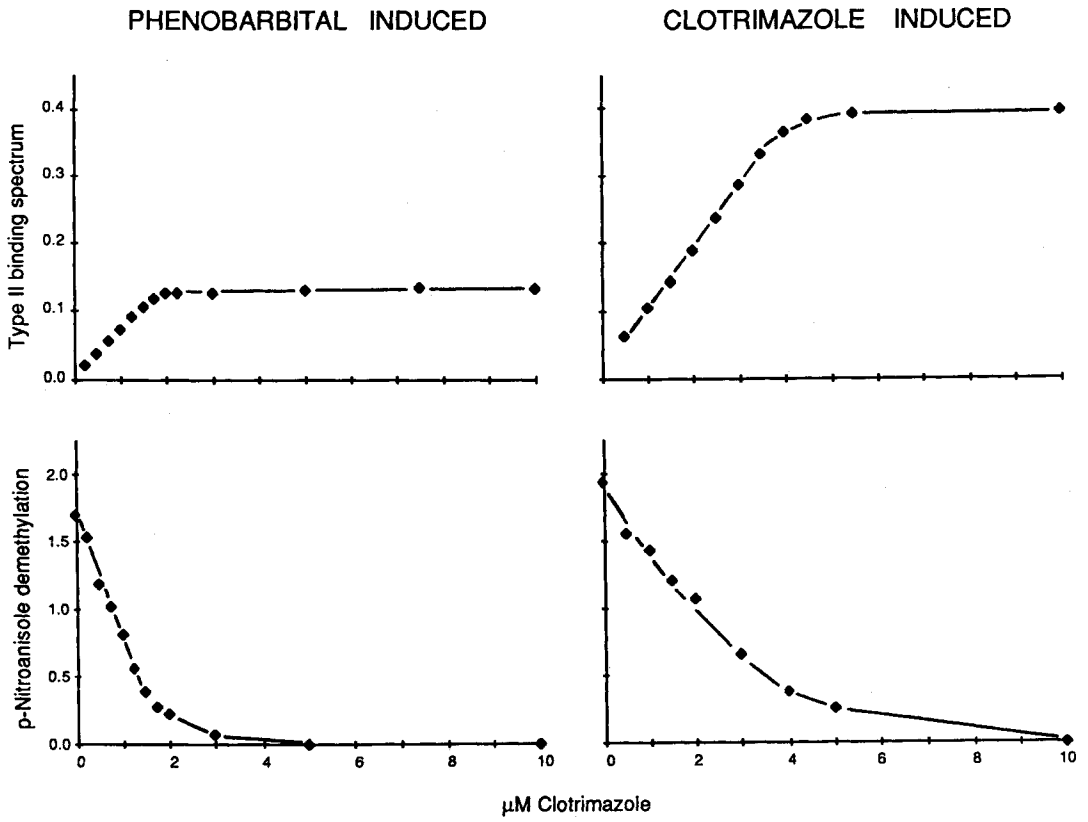

Fig. 1. Effect of clotrimazole concentration on the microsomal Type II difference spectrum and inhibition of *p*-nitroanisole demethylation. Clotrimazole was added to phenobarbital (left) and clotrimazole (right) induced microsomal suspensions (2 mg protein/ml), and the Type II difference spectrum (Δ absorbance 430–398 nm) was determined (upper panels). The ability of similarly treated microsomes to demethylate *p*-nitroanisole (nmol/mg/min) was also determined (lower panels). Cytochrome P-450 concentrations were 1.48 and 3.26 nmol/mg protein for phenobarbital- and clotrimazole-induced microsomes respectively.

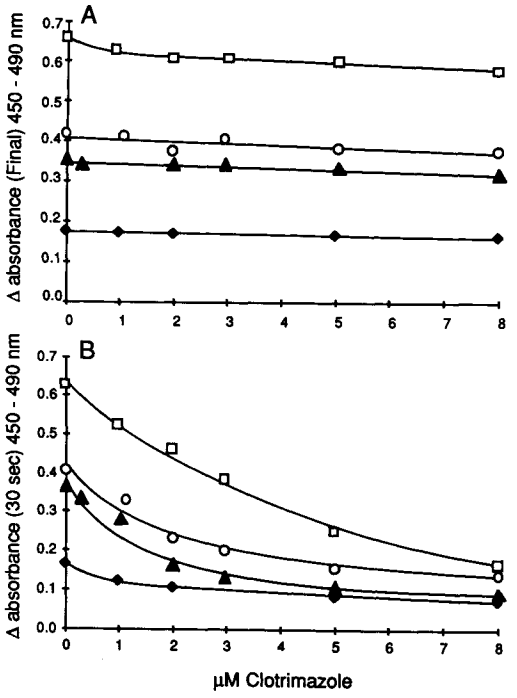

Fig. 2. Effect of clotrimazole *in vitro* on ferrous cytochrome P-450-carbon monoxide adduct formation. The amount of cytochrome P-450 CO-adduct formed 30 sec after the addition of dithionite to carbon monoxide gassed microsomal suspensions was determined (lower panel: B). The total amount finally formed was also monitored (upper panel: A). Microsomes were from clotrimazole-induced (□), phenobarbital-induced (○) dexamethasone-induced (▲) and uninduced (◆) rats. All were assayed at 2 mg protein/ml.

clotrimazole slowed the formation of the carbon monoxide [CO] adduct upon the chemical (dithionite) reduction of cytochrome P-450 in the presence of CO (Fig. 2B). Only the rate of formation was slowed, since the amount of cytochrome P-450 finally detectable as the CO complex (i.e. after prolonged time periods) was independent of the clotrimazole concentration (Fig. 2A). Thus, the slowing of cytochrome P-450 CO-adduct formation can also be used as an indicator of the presence of clotrimazole and, in Fig. 3, the reason for the 48-hr period between the last dose of clotrimazole and the time at which the microsomes were routinely prepared (Table 1) is readily apparent. It is the optimal time when the clotrimazole is no longer present in the microsomes (no slowing of cytochrome P-450 CO-adduct formation and inhibition of *p*-nitroanisole and erythromycin demethylations) and before decay of the induced cytochrome P-450 occurs. The clotrimazole "microsomal clearance time" was about 40 hr as judged by inhibition of cytochrome P-450 CO-adduct formation and inhibition of the demethylations (Fig. 3). Neither the total cytochrome P-450 nor NADPH cytochrome *c* reductase activity was affected by the presence of clotrimazole in the microsomes.

DISCUSSION

Clotrimazole was an extremely efficacious inducer of hepatic cytochrome P-450, producing concentrations that exceeded those of all but a few known inducing agents. The cytochrome induced showed no shift in CO absorbance maximum from controls and the microsomes showed monooxygenase activities similar to those seen after either phenobarbital

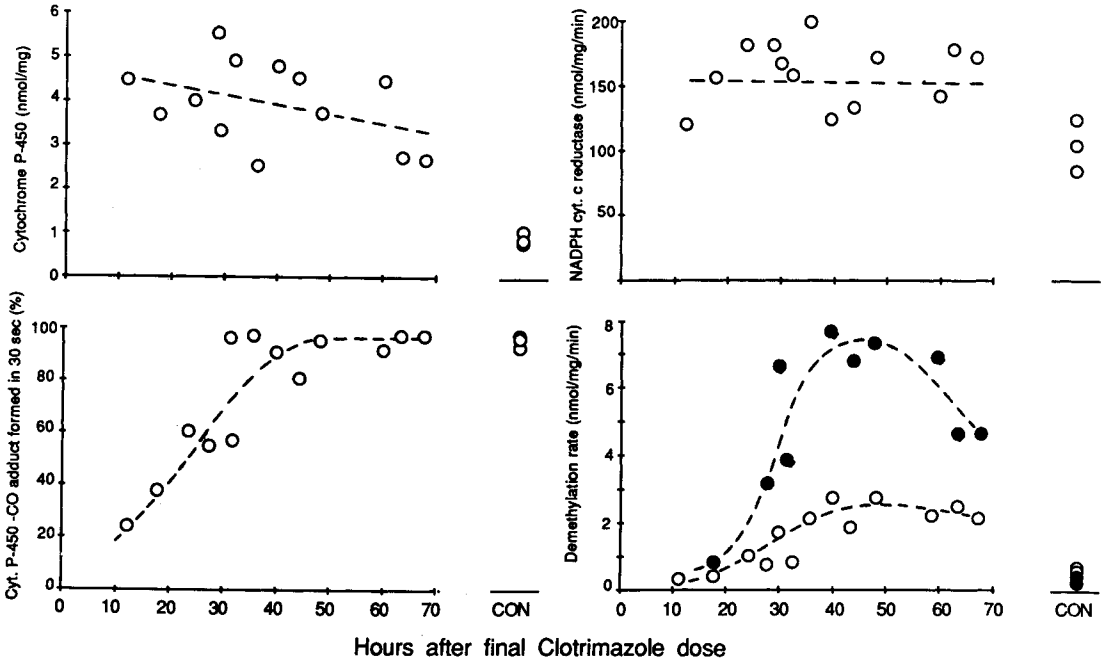

Fig. 3. Effect of time between clotrimazole injection and microsome preparation on expressed activities. Rats were treated for 3 days with clotrimazole (75 mg/kg/day), and the livers were removed at various times following the third injection. Five microsomal parameters were determined; each point was derived from a single microsomal preparation. Values from untreated rats (CON) are shown on the right of each panel. Demethylation reactions (lower right) were *p*-nitroanisole (○) and erythromycin (●).

or dexamethasone treatment. Since these two agents are known to induce isozymes of cytochrome P-450 to different extents, clotrimazole is either an inducer that induces in a mixed manner, or induces a new isozyme which catalyzes activities previously attributed to two different isozymes, but in a less efficient manner (lower turnover number). However, some monooxygenase activities that were induced by phenobarbital and dexamethasone were not increased by clotrimazole. The question of whether a new isozyme is induced could be resolved by the use of known isozyme specific antibodies.

Whether the powerful inducing properties of clotrimazole are related to the potent inhibitory properties remains to be elucidated. The commonality between clotrimazole and those nitrogenous compounds capable of forming cytochrome P-450 metabolic-intermediate complexes *in vivo* (SKF 525-A and troleandomycin), which are also powerful inducers, is their ability to inhibit monooxygenase activity *in vivo* for long time periods. The so far unsubstantiated idea of monooxygenase inhibition causing the accumulation of an endogenous inducer which is normally metabolized by cytochrome P-450 still remains a possibility.

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REFERENCES

1. K. W. Liebman and E. Ortiz, *Drug. Metab. Dispos.* **1**, 775 (1973).
2. C. F. Wilkinson, K. Hetnarski and L. J. Hicks, *Pestic. Biochem. Physiol.* **4**, 299 (1974).
3. J. J. Sheets and J. I. Mason, *Drug Metab. Dispos.* **12**, 603 (1984).
4. J. J. Heusner and M. R. Franklin, *Pharmacology* **30**, 266 (1985).
5. C. G. Meredith, A. L. Maldonado and K. V. Speeg Jr., *Drug Metab. Dispos.* **13**, 156 (1985).
6. R. Kahl, D. E. Freiderici, G. F. Kahl, W. Ritter and R. Krebs, *Drug Metab. Dispos.* **8**, 191 (1980).
7. D. S. Loose, P. B. Kan, M. A. Hirst, R. A. Marcus and D. Feldman, *J. clin. Invest.* **71**, 1495 (1983).
8. J. J. Sheets, J. I. Mason, C. A. Wise and R. W. Estabrook, *Biochem. Pharmac.* **35**, 487 (1986).
9. J. L. Riviere, *Pestic. Biochem. Physiol.* **19**, 44 (1983).
10. M. Murray, C. F. Wilkinson and K. Hetnarski, *Toxic. Lett.* **25**, 191 (1985).
11. G. A. Dannan, F. P. Guengerich, L. S. Kaminsky and S. D. Aust, *J. biol. Chem.* **258**, 1282 (1983).
12. A. Parkinson, S. H. Safe, L. W. Robertson, P. E. Thomas, D. E. Ryan, L. M. Reik and W. Levin, *J. biol. Chem.* **258**, 5967 (1983).
13. M. K. Buening and M. R. Franklin, *Drug Metab. Dispos.* **4**, 244 (1976).
14. D. Pessayre, V. Descatoire, M. Konstantinova-Mitcheva, J. C. Wandscheer, B. Colbert, R. Level, J. P. Benhameau, M. Jaouen and D. Mansuy, *Biochem. Pharmac.* **30**, 553 (1981).
15. V. Luu-The, J. Cumps and P. Dumont, *Biochem. biophys. Res. Commun.* **93**, 776 (1980).
16. S. A. Wrighton, P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young and P. S. Guzelian, *Biochemistry* **24**, 2171 (1985).
17. F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
18. R. A. Lubet, R. T. Mayer, J. W. Cameron, R. W. Nims, M. D. Burke, T. Wolff and F. P. Guengerich, *Archs Biochem. Biophys.* **238**, 43 (1985).
19. M. R. Franklin and R. W. Estabrook, *Archs Biochem. Biophys.* **143**, 318 (1971).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
22. Z. Liu and M. R. Franklin, *Archs Biochem. Biophys.* **241**, 397 (1985).
23. M. R. Franklin, *Molec. Pharmac.* **10**, 975 (1974).
24. T. Nash, *Biochem. J.* **55**, 416 (1953).
25. K. J. Netter and G. Seidel, *J. Pharmac. exp. Ther.* **146**, 61 (1964).
26. A. V. Klotz, J. J. Stegeman and C. Walsh, *Analyt. Biochem.* **140**, 138 (1984).
27. Y. Imai and R. Sato, *J. Biochem., Tokyo.* **62**, 239 (1967).
28. D. J. Waxman, G. A. Dannan and F. P. Guengerich, *Biochemistry* **24**, 4409 (1985).
29. B. S. S. Masters, J. Baron, W. E. Taylor, E. L. Isaacson and J. LoSpalluto, *J. biol. Chem.* **246**, 4143 (1971).