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EVALUATION OF MONOOXYGENASE INDUCTION AS A MEANS OF ENHANCING THE YIELD OF THE RAT LIVER CYTOCHROME P-450 ISOENZYME WITH HIGH AFFINITY FOR CIMETIDINE

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Abstract—The binding of cimetidine to liver microsomes prepared from untreated rats and rats pretreated with phenobarbitone or 3-methylcholanthrene has been investigated by difference spectroscopy and equilibrium partition methods. In M/15 phosphate buffer, pH 7.9, microsomes from each group of rats yielded markedly biphasic spectral binding curves, which have been interpreted in terms of two independent classes of cytochrome P-450 site with widely differing binding affinities for the drug. Support for such interpretation was provided by the finding that the spectral binding curve for a purified sample of the principal cytochrome P-450 isoenzyme from liver microsomes of phenobarbitone-pretreated rats could be described adequately by a single rectangular hyperbolic relationship, the spectral dissociation constant being indistinguishable experimentally from that for the weaker class of cytochrome P-450 binding site in the corresponding microsomes. The spectral dissociation constants were 2 μM and 80 μM for microsomes from untreated rats; 44 μM and 540 μM for those from phenobarbitone-pretreated rats; and 34 μM and 540 μM for microsomes from rats pretreated with 3-methylcholanthrene. On this basis, both classes of P-450 site in the microsomes from rats subjected to either pretreatment exhibited lower affinity for cimetidine than their counterparts in microsomes from untreated rats. Equilibrium partition studies of the higher-affinity class of microsomal binding site for cimetidine showed that the twofold increase in the cytochrome P-450 content of microsomes effected by 3-methylcholanthrene pretreatment was more than offset by a diminished proportion of the total cimetidine-binding capacity present as the higher-affinity, pharmacologically significant, receptor (18%, cf. 48% in control microsomes); and that phenobarbitone pretreatment resulted in replacement of the high-affinity receptor by one with a threefold weaker cimetidine-binding affinity. Thus the use of these monooxyginase inducers to enhance the cytochrome P-450 content of liver microsomes would seem to offer little potential in the isolation of the isoenzyme with high affinity for cimetidine.

Equilibrium partition and difference spectroscopy studies have recently shown normal rat liver microsomes to contain independent classes of cytochrome P-450 species exhibiting low (about 5 μM) and high (about 100 μM) apparent dissociation constants with respect to the binding of the histamine H_2-receptor antagonist, cimetidine [1]. That demonstration of a strong interaction between cimetidine and microsomal P-450 was of value in explaining the drug's apparent potency *in vivo* as a monooxygenase inhibitor [2–10], since in normal therapeutic use its maximal systemic concentration is in the 4–10 μM range [11, 12], and previous *in vitro* studies seemingly show cimetidine to be a rather poor inhibitor of microsomal monooxygenase [13–18].

From the pharmacological viewpoint, more detailed studies of monooxygenase inhibition would be greatly facilitated by the use of purified preparations of the cytochrome P-450 pigment with high binding affinity for cimetidine. Although cytochrome P-450-dependent monooxygenase activity is higher in liver than in other tissues [19], the absolute concentration of the pigment is nevertheless low, and pretreatment of experimental animals with a variety of inducing agents has often been employed to increase the cytochrome P-450 content of liver and other tissues prior to isolation of particular isoenzymes [20, 21]. Since phenobarbitone is one of the most potent and commonly used inducing agents, rats pretreated with this drug were initially used as the source of liver microsomes for purification of the principal cytochrome P-450 species by the method of Guengerich and Martin [22]. These studies served to illustrate the feasibility of isolating an individual cytochrome P-450 isoenzyme without affecting its cimetidine-binding characteristics. However, this principal isoenzyme resulting from phenobarbitone-induction exhibited low binding affinity for the drug.

These findings prompted an appraisal of the effects of pretreatment of rats with monooxygenase inducers on cimetidine-binding by liver microsomes, difference spectrum and equilibrium partition methods being used for the comparison of untreated, phenobarbitone- and 3-methylcholanthrene-pretreated rats. In each case biphasic spectral binding curves were obtained, but both classes of binding site in microsomes from either of the pretreated groups showed lower affinity for cimetidine than did their counterparts in control microsomes. These spectral results, together with equilibrium partition studies of the stronger microsomal interaction with

cimetidine, lead to the conclusion that the use of phenobarbitone and 3-methylcholanthrene, each representative of a major class of monooxygenase inducer, to enhance the pigment content of rat liver, offers little potential in the isolation of the cytochrome P-450 isoenzyme with high affinity for cimetidine.

MATERIALS AND METHODS

Microsomes were prepared by the Netter [23] method from the livers of three groups of 6-week-old, male, random-outbred, Wistar rats that had been obtained from the University of Queensland Central Animal Breeding Facility. One group was left untreated, a second group received daily intraperitoneal injections of phenobarbitone (B.P. grade) at a dose of 80 mg/kg body weight for 5 days, and the third group received daily i.p. injections of a sterile solution of 3-methylcholanthrene (Sigma) dissolved in corn oil at a dose of 25 mg/kg body weight for 3 days. All groups were fasted for 24 hr prior to sacrifice by cervical section. Washed microsomes were then stored at −70° in the sucrose/EDTA/Tris buffer of Netter [23] until used. Measurements of the pigment and protein concentrations by the Estabrook *et al.* [24] and Lowry *et al.* [25] procedures, respectively, showed the specific cytochrome P-450 contents to be 0.7 nmole/mg protein for microsomes from control rats, 1.6 nmole/mg protein for microsomes from phenobarbitone-pretreated rats, and 1.2 nmole/mg protein for microsomes from rats subjected to pretreatment with 3-methylcholanthrene. Cimetidine was kindly donated by Smith Kline and French (Australia), and [*N*-methyl-^{3}H]cimetidine (TRK615; 23 Ci/mmole) was obtained from Amersham Australia Pty. Ltd. Other chemicals were of reagent grade.

The method of Guengerich and Martin [22] was used to purify the principal cytochrome P-450 isoemzyme (B2 fraction) from liver microsomes of rats pretreated with phenobarbitone. A value of 9.2 nmole/mg protein was found for the specific pigment content of the isolated isoenzyme, the protein estimation for this determination being based on the Peterson [26] adaptation of the Lowry [25] procedure to account for the effects of solubilizing agents, glycerol and detergents, on colour yield. A single haemoprotein band was observed in electrophoresis of the isolated material on SDS-polyacrylamide gels [27] stained by the Høyer-Hansen [28] method.

Spectral studies of cimetidine binding. All spectral studies of the interaction of cimetidine with cytochrome P-450 preparations in M/15 phosphate buffer, pH 7.9, employed a pigment concentration of 1 nmole/ml and drug concentrations in the range 20 μM–3mM, the latter being achieved by 100-fold dilution of methanolic solutions of cimetidine. In order to verify that each of the cytochrome preparations yielded a 'type II' difference spectrum [29] in the presence of 1% methanol, an Aminco DW-2a spectrophotometer was used in the split-beam mode, the same concentration of methanol being included in the contents of the reference cuvette. Typical 'type II' difference spectra were obtained in all cases, the isosbestic and peak absorbance wavelengths being 421 and 432 nm for control microsomes, 416 and 430 nm for microsomes from phenobarbitone-pretreated rats, and 417 and 430 nm for those from animals pretreated with 3-methylcholanthrene. The purified cytochrome P-450 fraction (B2 peak) also showed a 'type II' difference spectrum with cimetidine, characterized by isosbestic and peak absorbance wavelengths of 416 and 430 nm, respectively. As in the previous investigation [1], spectral binding curves at 25°C were obtained by operating the spectrophotometer in dual wavelength mode using the appropriate pair of wavelength settings for each of the pigment preparations.

Equilibrium partition studies. Equilibrium partition studies of the binding of cimetidine to cytochrome P-450 preparations in M/15 phosphate buffer, pH 7.9, were carried out essentially in accordance with the procedure described previously [1], except that an approximately 10-fold higher pigment concentration was employed to increase the precision with which the extent of cimetidine binding could be determined. For control microsomes the protein content and range of cimetidine concentrations (suitably supplemented with trace quantities of [^{3}H]cimetidine) used were 12.6 mg/ml and 0.5–28 μM, respectively; for microsomes from phenobarbitone-pretreated rats the corresponding values were 8.3 mg/ml and 0.9–24 μM; and for microsomes from 3-methylcholanthrene-pretreated rats the relevant magnitudes were 9.7 mg/ml and 0.4–19 μM. Reaction mixtures were allowed to equilibrate at room temperature (23–25°C) for 15 min prior to centrifugation in a Beckman Airfuge (178,000 *g* for 5 min) to obtain a sample of the liquid phase for analysis in a Packard model C2425 liquid scintillation counter.

RESULTS

Figure 1 summarizes, in Scatchard [30] format, the results of studies designed to test the feasibility of isolating an individual cytochrome P-450 isoenzyme without affecting its cimetidine-binding characteristics. The essential linearity of Fig. 1(a), which refers to the purified cytochrome P-450 isoenzyme of liver microsomes from phenobarbitone-pretreated rats, signifies that the spectral binding results for this isolated P-450 fraction are described adequately by a rectangular hyperbolic relationship. Indeed, non-linear regression analysis of the untransformed data by the method used previously [1] confirmed that a single rectangular hyperbolic relationship sufficed for their description: the magnitudes of the apparent dissociation constant (K_S) and the absorbance change associated with stoichiometric complex formation (ΔA_m) are listed in the first line of Table 1. These results for a cytochrome P-450 fraction which yields a single haemoprotein band in SDS gel electrophoresis are entirely consistent with our earlier conclusion [1] that site heterogeneity is responsible for the curvilinearity of Scatchard plots for the binding of cimetidine to liver microsomes. In this regard it should be noted that the Scatchard plot of spectral results for microsomes from phenobarbitone-pretreated rats is curvilinear (Fig. 1b), the parameters obtained by non-linear regression analysis of the

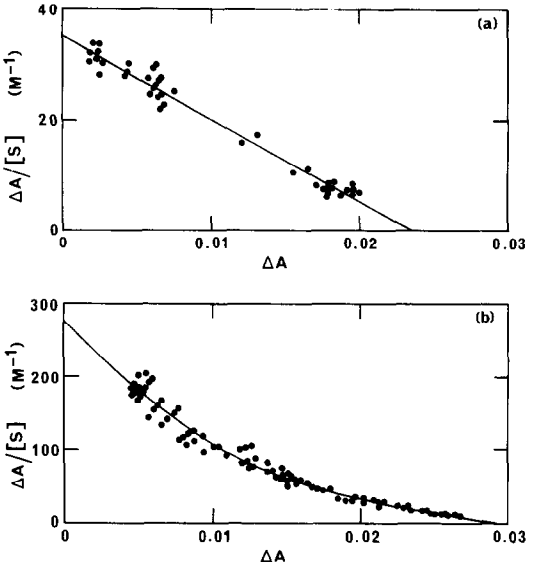

Fig. 1. Scatchard plots of spectral binding data on the interaction of cimetidine with (a) cytochrome P-450 (fraction B2) isolated from liver microsomes prepared from phenobarbitone-pretreated rats; and (b) liver microsomes prepared from phenobarbitone-pretreated rats: the interactions were studied at 25°C in M/15 phosphate buffer, pH 7.9

untransformed results in terms of two rectangular hyperbolae being summarized in the second line of Table 1.

Comparison of the values presented in the first two lines of Table 1 shows that the apparent spectral dissociation constant for the isolated cytochrome P-450 isoenzyme (K_s = 660 μM) is not significantly different ($P > 0.5$) from that found for the class of microsomal site with lower cimetidine-binding affinity (K_s = 540μM). Thus purification of this cytochrome P-450 species has not significantly altered its cimetidine-binding characteristics. Such a result, it should be noted, was not necessarily to be expected, since the identical pigment fraction [22] has been shown [31] to have a Michaelis constant (K_m) for 7-ethoxycoumarin (27 μM) which was markedly different from either of the two K_m values (7.2 μM and 148 μM) obtained for oxidation of this substrate by the parent microsomal preparation.

Three additional points should be considered in relation to these results. (i) The total absorbance change of 0.029 (for 1 μM pigment) observed with microsomes from phenobarbitone-pretreated rats is significantly greater ($P < 0.01$) than the corresponding value (0.023) for the isolated isoenzyme, a factor which points to the sensitivity of the spectral change to environmental factors. (ii) Although the attempt to purify a cytochrome P-450 isoenzyme with unchanged cimetidine-binding characteristics has met with success, the isolated isoenzyme is a form of cytochrome P-450 with no pharmacological relevance in the present context, since the peak systemic concentration of cimetidine in normal clinical use is only in the 4–10 μM range. (iii) The apparent spectral dissociation constants reported in line 2 of Table 1 are much higher than the values of 2 μM and 104 μM reported previously [1] for control rat liver microsomes, a finding which suggests that induction of cytochrome P-450 synthesis by phenobarbitone impairs the cimetidine-binding affinity of the resulting microsomes. This aspect of cytochrome P-450 induction was accordingly investigated further.

Spectral binding results on the interaction of cimetidine with liver microsomes from untreated and 3-methylcholanthrene-pretreated rats are presented as Scatchard plots in Figs. 2(a) and 2(b), respectively. These plots are also curvilinear but differ in the detailed form of their curvilinearity both from each other and from that seen (Fig. 1b) for microsomes from phenobarbitone-pretreated rats. This comparison was made quantitative by regression analysis of the untransformed results in terms of two rectangular hyperbolae (lines 2–4 in Table 1). In this regard the first point to be noted is that the present values of 2 μM and 79 μM (line 3 of Table 1) for the apparent spectral dissociation constants describing the interactions of cimetidine with control microsomes are in good agreement with the corresponding values (2 μM, 104 μM) reported previously [1] for a similar preparation. Secondly, from the final line of Table 1 it is evident that pretreatment of rats with 3-methylcholanthrene gave results which parallelled those observed with phenobarbitone pretreatment in that both classes of microsomal binding site exhibit impaired affinity for cimetidine. Inspection of Table 1 also reveals marked differences between the maximal spectral changes (ΔA_m; referred to 1 μM cytochrome P-450) for the various interactions; but as noted above in regard to Fig. 1(b), the sensitivity of such spectral differences to environmental factors precludes their use as a reliable guide to the relative distribution of microsomal cytochrome P-450

Table 1. Spectral studies of the interaction between cytochrome P-450 and cimetidine at 25°C in M/15 phosphate buffer, pH 7.9

Pretreatment	P-450 sample	Stronger interaction		Weaker interaction	
		K_s (μM)*	ΔA_m*	K_s (μM)*	ΔA_m*
Phenobarbitone	B2 fraction	—	—	660 (± 30)	0.023 (± 0.001)
Phenobarbitone	Microsomes	44 (± 12)	0.011 (± 0.003)	540 (± 170)	0.018 (± 0.002)
None	Microsomes	2 (± 3)	0.002 (± 0.001)	79 (± 12)	0.013 (± 0.001)
3-Methylcholanthrene	Microsomes	34 (± 8)	0.007 (± 0.001)	540 (± 190)	0.008 (± 0.001)

* Numbers in parentheses denote the uncertainty (twice the S.E.M.) associated with the magnitude of the parameter obtained by nonlinear regression analysis.

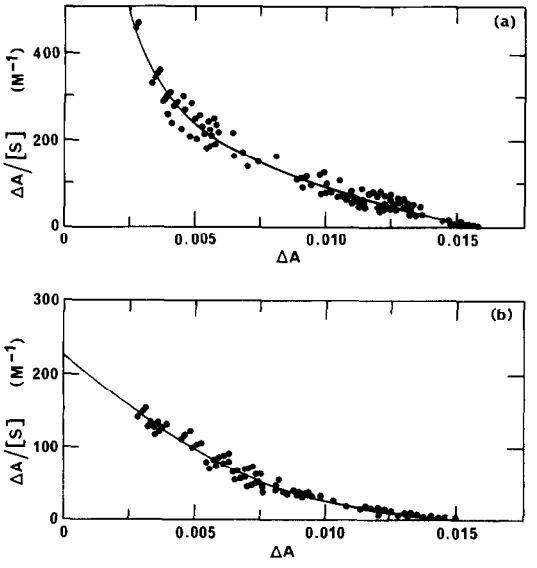

Fig. 2. Scatchard plots of spectral binding data on the interaction of cimetidine with liver microsomes prepared from (a) untreated rats and (b) 3-methylcholanthrene-pretreated rats under the conditions pertaining to Fig. 1.

between the two classes of site. For this purpose use has therefore been made of equilibrium partition studies [1], which also provide a more definitive description of the stronger interaction between cimetidine and these microsomal preparations.

Results of equilibrium partition studies employing low concentrations of cimetidine ($< 30\ \mu$M) and microsomes from untreated (●), phenobarbitone-pretreated (■) and 3-methylcholanthrene-treated (○) rats are summarized in Fig. 3, where the linear relationships correspond to the single rectangular hyperbolae that best describe the three sets of untransformed results. In this regard it is noted that the upward curvature of the Scatchard plot at low r values for control microsomes could well indicate the existence of sites with even higher affinity for cimetidine; but that their existence is not unequivocally established by these results. Regression

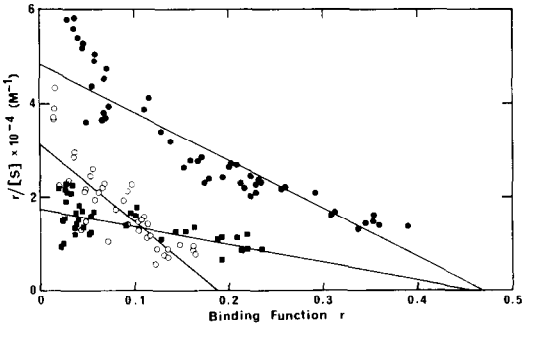

Fig. 3. Scatchard plots of equilibrium partition binding data on the interaction of cimetidine with microsomes prepared from untreated rats (●), phenobarbitone-pretreated rats (■) and rats pretreated with 3-methylcholanthrene (○), the interactions being studied at 23–25°C in M/15 phosphate buffer, pH 7.9.

analysis of the data in terms of two rectangular hyperbolae signifies a dissociation constant of 0.3 (± 0.3) μM for such sites, which, if present, comprise only 2–6% of the binding capacity. However, the curvature could also reflect underestimation of cimetidine at the low end (0.5–1.0 μM) of the concentration range investigated. Experiments with cimetidine alone have indicated a recovery of 98 ± 2% of cimetidine, in this concentration range, from the polyallomer tubes used to contain the reaction-mixtures: any such adsorption becomes of decreasing significance with increasing cimetidine concentration. We therefore take the view that each set of results is appropriately described by the linear relationship shown in Fig. 3. For control microsomes the present dissociation constant, K_d, of 9.8 (± 1.3) μM compares very favourably with the earlier value [1] of 8.3 (± 1.3) μM; and also with the apparent spectral dissociation constant, K_s, for the stronger interaction (Table 1). The abscissa intercept of 0.47 is also in reasonable agreement with the earlier estimate [1] of 0.58 for the fraction of microsomal sites with this high affinity for cimetidine. Essentially the same proportion of high affinity sites (0.46 cf. 0.47) was found for microsomes from rats pretreated with phenobarbitone, but the dissociation constant for the interaction, 26 (± 4) μM, was significantly higher. On the other hand, pretreatment of rats with 3-methylcholanthrene gave rise to microsomes with essentially unchanged cimetidine-binding affinity (K_d = 6.1 (± 0.7) μM) but with a markedly decreased proportion (0.18) of sites showing these characteristics.

From the viewpoint of isolating the cytochrome P-450 pigment with high cimetidine-binding affinity the use of either of these inducers to enhance the yield of microsomal pigment is clearly unsatisfactory. Phenobarbitone pretreatment is precluded on the grounds that the high-affinity class of receptor has seemingly been replaced by a class of receptor with a threefold lower affinity for cimetidine; and although this criticism does not appear to apply to 3-methylcholanthrene induction (at least on the basis of the equilibrium partition studies), the twofold increase in microsomal pigment content effected by such pretreatment is more than offset by the decreased proportion (18%, cf. 48%) of cimetidine-binding capacity present as the high-affinity class of receptor site.

DISCUSSION

In this investigation of the effectiveness of inducers in studies aimed at isolating the microsomal cytochrome P-450 isoenzyme with high cimetidine-binding affinity, phenobarbitone and 3-methylcholanthrene were selected as exemplars of the two principal classes of monooxygenase inducer [32], which apparently act by different mechanisms [33] and evoke markedly different responses in monooxygenase-dependent metabolism of a wide variety of substrates [34]. However, the use of both of these inducers has led to unacceptable changes in the cimetidine-binding characteristics of the microsomes, a result that has been interpreted as indicating the selective production of either new or

previously minor cytochrome P-450 species rather than uniform enhancement of the amounts of all components present in control microsomes. In this regard there are also several other lines of evidence which indicate that phenobarbitone pretreatment of rats causes the production of a number of polypeptides [35, 36] as the result of increased production of new, translationally active mRNA [37]. Furthermore, it has been shown recently [38] that the principal cytochrome P-450 isoenzyme whose synthesis is induced in rat liver by phenobarbitone pretreatment constitutes only a small proportion of total liver cytochrome P-450 content of control animals. The induction effect is thus indeed not uniform, a factor borne out by the present results in that microsomes from phenobarbitone-pretreated rats showed no evidence of the binding characteristics associated with either receptor population in control microsomes. Although information concerning the mechanism of the inductive effect of 3-methylcholanthrene is less complete than that relating to phenobarbitone, it does appear that the principal cytochrome P-450 isoenzyme induced by pretreatment with this hydrocarbon also represents only a small proportion of the total hepatic pigment in control animals [38]. The decreased proportion of total cimetidine-binding capacity present as the high-affinity form (Fig. 3) may also be taken to indicate that 3-methylcholanthrene induction has led to the preferential synthesis of a population of polypeptides other than that with high affinity for the drug.

From the difference spectrum studies with the major cytochrome P-450 isoenzyme isolated from hepatic microsomes from phenobarbitone-pretreated rats it is clear that the purification procedures involved led to no significant alteration of its binding affinity for cimetidine. Although this protein was of no pharmacological relevance in that it represented a class of microsomal cytochrome P-450 with low binding affinity, its isolation in a form with unimpaired ligand-binding characteristics does hold promise for studies designed to purify the isoenzyme with high cimetidine-binding affinity. In relation to earlier purification studies it is noteworthy that maximization of specific pigment content has frequently been the guiding determinant in the isolation of cytochrome P-450 isoenzymes [20, 21]; and that although some of these preparations proved to have enhanced catalytic activity in comparison with the parent microsomes [39], others exhibited unchanged or even diminished activity in this respect [40]. Only recently has the value of assessing the isolation procedure on the basis of the specific activity of the resulting preparation been emphasized [41]. The present study highlights the utility of another criterion, namely, the ligand-binding affinity of the purified material.

In summary, this investigation has yielded two major findings. First, it is possible to isolate a microsomal cytochrome P-450 isoenzyme with unchanged cimetidine-binding characteristics. Secondly, the use of monooxygenase inducers to enhance the cytochrome P-450 content of liver microsomes seems to offer little potential in the isolation of isoenzymes with pharmacological relevance in normal animals, since pretreatment with exemplars of the two major classes of monooxygenase inducer has not led to increased amounts of the required cytochrome P-450 isoenzyme with high cimetidine-binding affinity.

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