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NADPH-CYTOCHROME P-450 REDUCTASE: PREFERENTIAL INHIBITION BY ELLIPTICINE AND OTHER TYPE II COMPOUNDS HAVING LITTLE EFFECT ON NADPH-CYTOCHROME *c* REDUCTASE

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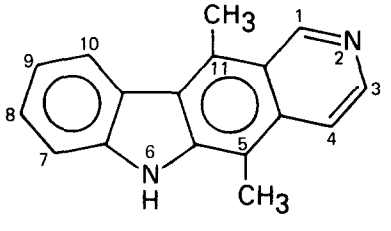
Abstract—Ellipticine (5,11-dimethyl-[6H]-pyrido[4,3b]carbazole) binds with an affinity greater than most other compounds known to interact with P-450. Control and 3-methylcholanthrene-induced aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (EC 1.14.14.2) and acetanilide 4-hydroxylase and control and phenobarbital-induced ethylmorphine *N*-demethylase activities are all markedly inhibited by ellipticine to about the same extent. Ellipticine and other Type II compounds (metyrapone, octylamine-1, pyridine and aniline) preferentially inhibit NADPH-cytochrome P-450 reductase activity, while affecting NADPH-cytochrome *c* reductase activity very little. Butanol-1, a compound having pure Reverse Type I character, does not block P-450 reductase activity like these Type II compounds. These data suggest that Type II compounds bind to P-450 ferric iron in the sixth coordinate position in such a way as to impede transfer of the first electron from the hydrophobic binding site of the reductase to the P-450–substrate complex, while leaving unencumbered any transfer of electrons from the hydrophilic binding site of the reductase to soluble electron acceptors such as cytochrome *c*. These data indicate that ellipticine may be very useful in attempting to understand the mechanism by which electrons are transferred from the reductase to the cytochrome(s) P-450.

Ellipticine (5,11-dimethyl-[6H]-pyrido[4,3b] carbazole) (Fig. 1) and its 9-methoxy derivatives are plant alkaloids from the genus *Ochrosia* that have strong antineoplastic activity against murine leukemia L1210 and several solid tumors [1,2]. The 9-methoxy derivative has been partially successful in treating human acute myeloid leukemia [3]. The 9-hydroxy and 7-hydroxy derivatives appear to be more active against murine leukemia L1210 than ellipticine or the 9-methoxy parent compound [4], implicating the potential importance of (one or more forms of) cytochrome P-450 in producing the active antitumor metabolite.



Ellipticine

Fig. 1. Chemical structure of ellipticine.

Ellipticine and some of its derivatives bind strongly to P-450, eliciting typical Type II difference spectra with apparent K_s values of less than 1 μM [5]. Moreover, these chemicals block aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity, the covalent binding of benzo[*a*]pyrene metabolites to DNA, and benzo[*a*]pyrene mutagenesis in the *Salmonella*/liver assay *in vitro* [5]. The apparent K_s (approximately 0.43 μM) for ellipticine bound to control rat liver microsomal P-450 is surprisingly lower than that for ellipticine bound to liver microsomal P-450 from rats treated with benzo[*a*]pyrene, Aroclor 1254, or phenobarbital. Further, ellipticine is a better inhibitor (by more than 10-fold) of aryl hydrocarbon hydroxylase activity than metyrapone or 7,8-benzoflavone in liver microsomes from rats regardless of pretreatment: control untreated, or benzo[*a*]pyrene, Aroclor 1254, or phenobarbital [5]. These data suggest to us a somewhat unique interaction between ellipticine and many (or all) forms of P-450. This report, therefore, extends those studies by Lesca and coworkers. Through the use of this very potent inhibitor of P-450 reduction, we were able to extrapolate our findings to many other Type II compounds having poorer affinity for the P-450 reductase than ellipticine and to arrive at a general concept for distinguishing between "NADPH-cytochrome P-450 reductase activity" and "NADPH-cytochrome *c* reductase activity".

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MATERIALS AND METHODS

Materials. Benzo[*a*]pyrene, 3-MC,* NADPH, octylamine-1, and cytochrome *c* (Type III, horse heart) were purchased from the Sigma Chemical Co, (St. Louis, MO); NADPH from Boehringer-Mannheim Biochemicals (Indianapolis, IN); pyridine from the Baker Chemical Co. (Phillipsburg, NJ); metyrapone from the Aldrich Chemical Co. (Milwaukee, WI); aniline and acetanilide from the Eastman Kodak Co. (Rochester, NY); ethylmorphine and sodium phenobarbital from Merck & Co. (Rahway, NJ); [^{14}C]acetanilide (uniformly labeled in the ring, 11 mCi/mmole) from California Bionuclear (Sun Valley, CA); generally tritiated 4-hydroxyacetanilide[^{3}H] (400 mCi/mmole) from New England Nuclear (Boston, MA); and generally tritiated [^{3}H]benzo[*a*]pyrene (20 Ci/mmole) from the Amersham/Searle Corp. (Arlington Heights, IL). Ellipticine was a gift from Mr. Leonard H. Kedda, Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD). The remainder of the materials was obtained from sources cited previously [6,7]. Benzo[*a*]pyrene, acetanilide, and 4-hydroxyacetanilide[^{3}H] were recrystallized prior to use [8,9]. B6 and D2 mice were obtained from the Veterinary Resources Branch, National Institutes of Health Animal Supply (Bethesda, MD). All animals were weanlings of either sex.

Treatment of mice and preparation of microsomes. 3-MC in corn oil was administered as a single intraperitoneal dose (200 mg/kg) 2 days before killing. Phenobarbital in 0.85% NaCl was given as intraperitoneal doses of 30 mg/kg the first day and 60 mg/kg the second and third days; the mice were killed 24 hr after the third dose. Untreated controls received either corn oil (50 ml/kg) or 0.85% NaCl (25 ml/kg) within the same time frame; in several experiments, no differences in the properties of either microsomal reductase were found under either control condition. Liver microsomes in 30% glycerol–0.25 M potassium phosphate buffer, pH 7.25, were prepared exactly as described in detail [10]. Freezing the microsomal pellet at −80° for 1 week or longer before use did not affect the results.

Enzyme assays. Aryl hydrocarbon hydroxylase [8], acetanilide 4-hydroxylase [9], ethylmorphine *N*-demethylase [11], NADPH-cytochrome P-450 reductase [12], and NADPH-cytochrome *c* reductase [13] activities were determined by the methods described in the references cited. An Aminco-Chance DW-2 recording spectrophotometer was used with the dual-beam mode for the P-450 reductase (450 vs 490 nm) and the cytochrome *c* reductase (550 vs 557.2 nm) assays.

Spectrophotometry. Total P-450 content was determined as usual [14], with an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the difference in absorption between the Soret maximum around 450 nm and the baseline at 490 nm. Difference spectra were obtained by the original method described by Remmer *et al.* [15].

* Abbreviations used are: 3-MC, 3-methycholanthrene; B6, the inbred C57BL/6N mouse strain; and D2, the inbred DBA/2N mouse strain.

Binding of radiolabeled benzo[a]*pyrene metabolites to DNA.* The techniques for (i) incubation of liver microsomes with NADPH, deproteinized salmon sperm DNA, and [^{3}H]benzo[*a*]pyrene, (ii) reisolation of the DNA, (iii) digestion of the DNA with DNase I, phosphodiesterase type II, and alkaline phosphatase type II, and (iv) the purification of benzo[*a*]pyrene metabolite–nucleoside complexes eluted by Sephadex LH20 column chromatography were all performed exactly as described previously [7].

Addition of inhibitors to enzyme assays. Ellipticine in dimethylsulfoxide, and metyrapone, octylamine-1, or pyridine in acetone, and aniline HCl in water were added in minimal amounts to the enzyme assays. Control 100 per cent starting activity denotes in all cases the enzyme activity to which the minimal amount of vehicle alone had been added. Each experiment was performed three times to ensure reproducibility; a typical experiment is illustrated.

RESULTS

Inhibitory effect of ellipticine on three mono-oxygenase activities. Ellipticine markedly inhibited, to about the same extent, aryl hydrocarbon hydroxylase, acetanilide 4-hydroxylase, and ethylmorphine *N*-demethylase activities (Fig. 2). The unusual aspect of this finding is that there was no preferential inhibition by ellipticine of control versus 3-MC-induced aryl hydrocarbon hydroxylase or acetanilide 4-hydroxylase activity. Moreover, it is unusual that control and phenobarbital-induced ethylmorphine

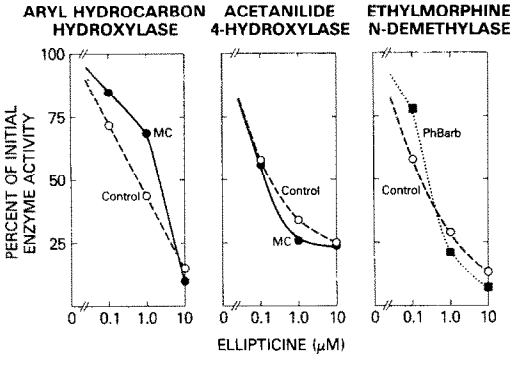

Fig. 2. Effects of ellipticine *in vitro* on three liver microsomal mono-oxygenase activities from control, 3-methylcholanthrene-treated (MC), or phenobarbital-treated (PhBarb) B6 mice. The initial "100 per cent activity" for aryl hydrocarbon hydroxylase was 470 and 2280 pmoles of 3-hydroxybenzo[*a*]pyrene formed/min/mg of protein in control and 3-methylcholanthrene-treated mice respectively. The "100 per cent activity" for acetanilide 4-hydroxylase was 610 and 2900 pmoles of 4-hydroxyacetanilide formed/min/mg of protein in control and 3-methylcholanthrene-treated mice respectively. The "100 per cent activity" for ethylmorphine *N*-demethylase was 11 and 38 nmoles of formaldehyde formed/min/mg of protein in control and phenobarbital-treated mice respectively. The appropriate amount of ellipticine was added in 10 μl dimethylsulfoxide to the usual 1-ml reaction mixture, and the mixture was incubated at 37° for 1 min prior to addition of the substrate to start the reaction; samples not containing ellipticine received 10 μl dimethylsulfoxide alone.

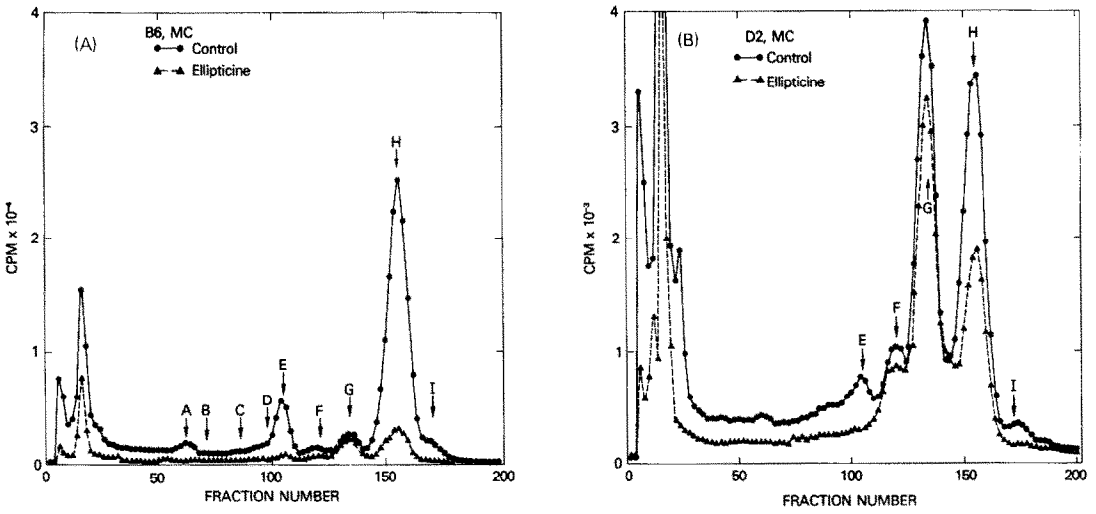

Fig. 3. Effects of ellipticine on benzo[*a*]pyrene metabolites covalently bound to DNA, as shown by a Sephadex LH20 column chromatogram of enzymically digested DNA. Microsomes (4 mg protein) from six combined livers of 3-methylcholanthrene(MC)-treated B6 (panel A) or D2 (panel B) mice were incubated for 30 min at 37° in a total volume of 10 ml containing NADPH and an NADPH-regenerating system, 20 mg of deproteinized salmon sperm DNA, and 60 nmoles of [^{3}H]benzo[*a*]pyrene [7]. The DNA was then reisolated and digested to nucleosides by the method described in detail [7]. Peaks A (most polar) through I (least polar) were eluted by a 30–100% methanol in water gradient, and those benzo[*a*]pyrene metabolites known to contribute to each of these nine peaks have been tentatively identified [7, 17]. The nucleoside(s) component in each peak is unknown, except that peak E is known to contain principally benzo[*a*]pyrene 7,8-diol-9,10-epoxide bound via the ^{2}N of guanine [18; reviewed in Ref. 19]. The possible sources of the radioactivity eluted prior to fraction No. 40 are not understood and have been discussed elsewhere [7].

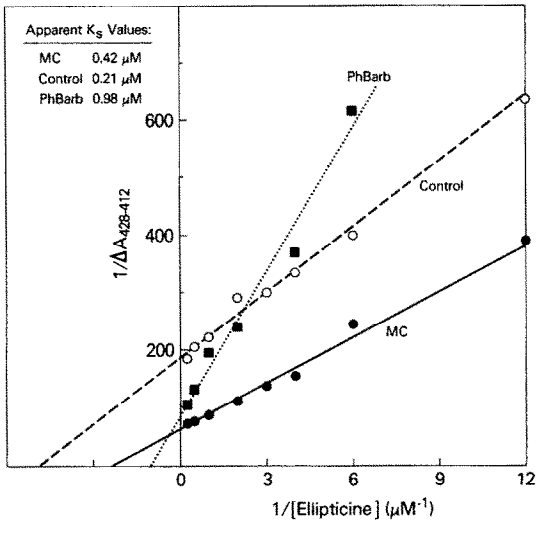

Fig. 4. Lineweaver–Burk plot of the peak-to-trough height ($\Delta A_{428-412}$) of the Type II difference spectrum as a function of ellipticine concentration in liver microsomes from control, 3-methylcholanthrene-treated (MC), or phenobarbital-treated (PhBarb) B6 mice. Between 10 and 50 μl dimethylsulfoxide containing ellipticine were added to the 2.5 ml in the sample cuvette, and a corresponding amount of dimethylsulfoxide alone was added to the reference cuvette. The same maximal spectra effect was obtained by the noncumulative addition of a highly concentrated solution of ellipticine in dimethylsulfoxide. The microsomal protein concentration was between 0.5 and 1.0 mg/ml.

N-demethylase activities were inhibited as much as the aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activities. Control aryl hydrocarbon hydroxylase activity and 3-MC-induced aryl hydrocarbon hydroxylase activity are catalyzed by different forms of P-450, and this fact appears to be true also for control and 3-MC-induced acetanilide 4-hydroxylase activity (discussed in detail in review, Ref. [16]).

Inhibition of DNA binding of benzo[a]*pyrene metabolites by ellipticine.* Since ellipticine had been shown [5] to block total covalent binding of benzo[*a*]pyrene metabolites to DNA, we wondered if any metabolite–nucleoside peaks would be preferentially inhibited (Figs. 3A and 3B). Peaks A through F and H and I comprise metabolites predominantly formed by 3-MC-inducible P_1-450 complexed with one or more nucleosides and, therefore, are much greater in the 3-MC-treated genetically responsive B6 mouse than the 3-MC-treated genetically nonresponsive D2 mouse [17]. Peak G represents metabolism by one or more forms of control P-450; this is principally the 4,5-oxide bound to one or more nucleosides [7] and thus is much greater in the D2 than in the 3-MC-treated B6 mouse [17]. Ellipticine appeared to block preferentially P_1-450-mediated metabolites which bind to DNA more than reactive metabolites catalyzed by control forms of P-450. Ellipticine blocked all benzo[*a*]pyrene metabolite formation to some extent, however. Therefore, it appears that ellipticine is acting at a site prior to

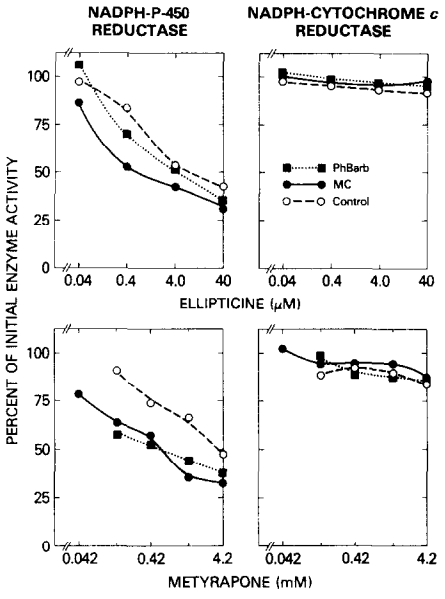

Fig. 5. Effects of ellipticine (top) or metyrapone (bottom) on the P-450 reductase (left) and the cytochrome *c* reductase (right) activities in liver microsomes from control, 3-methylcholanthrene-treated (MC), or phenobarbital-treated (PhBarb) B6 mice. For the P-450 reductase assay, ellipticine in 20 μl dimethylsulfoxide or metyrapone in 20 μl acetone was added to the reaction mixture prior to addition of the NADPH to initiate the 3-ml reaction in the experimental cuvette. For the cytochrome *c* reductase assay, 20 μl dimethylsulfoxide containing ellipticine and 20 μl acetone containing metyrapone were added to the reaction mixture prior to starting the 3-ml reaction by the addition of NADPH. After an appropriate preincubation time at room temperature (about 10–15 min, i.e. the same amount of time required to obtain anaerobic conditions for the P-450 reductase assay), the cytochrome *c* reduction rates were determined. At these concentrations of Type II compounds and during these time periods, it was determined that spectral changes caused by Type II compounds complexed with reduced P-450 did not interfere significantly with either reductase assay. Reference cuvettes received appropriate volumes of these solvents alone. Each symbol represents the mean of three experiments, with standard deviations of ± 15 per cent or less. The typical "100 per cent activity" for control, 3-methylcholanthrene-induced, and phenobarbital-induced P-450 reductase activity was 4.4, 5.0 and 8.0 nmoles P-450 reduced/mg of microsomal protein respectively. The typical "100 per cent activity" for control, 3-methylcholanthrene-induced, and phenobarbital-induced cytochrome *c* reductase was 42, 45 and 63 nmoles cytochrome *c* reduced/mg of microsomal protein respectively.

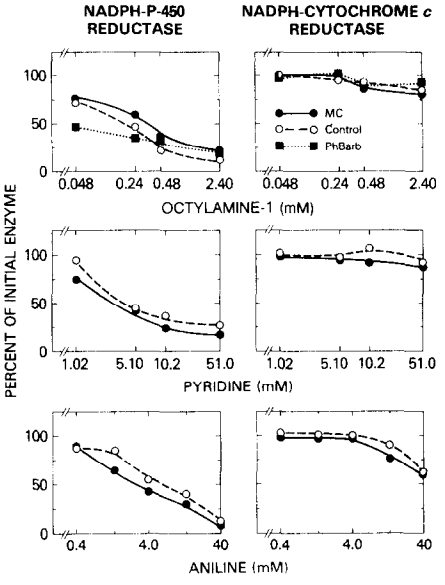

Fig. 6. Effect of octylamine-1 (top), pyridine (middle), or aniline (bottom) on the P-450 reductase (left) and the cytochrome *c* reductase (right) activities in liver microsomes from control, 3-methylcholanthrene-treated (MC), or phenobarbital-treated (PhBarb) B6 mice. Additional experimental details are provided in the legend to Fig. 5 and under 'Materials and Methods.'

the oxygenation of various substrates by the different forms of P-450.

Apparent binding constant between ellipticine and P-450. As had been true with rat liver microsomes [5], the apparent K_s value for control mouse liver microsomes (Fig. 4) was lower than that for 3-MC-induced microsomes, and both of these values were lower than that for phenobarbital-induced microsomes. These data indicate that certain differences do exist in the specific interaction between ellipticine and the numerous forms of control or inducible P-450.

Effects of ellipticine and other Type II compounds on P-450 reductase and cytochrome c reductase activities. Ellipticine at increasing concentrations *in vitro* blocked equally the P-450 reductase activity from control, 3-MC-treated, and phenobarbital-treated mice (Fig. 5), whereas cytochrome *c* reductase activity remained unaffected at these concentrations. Metyrapone inhibited the P-450 reductase activity from 3-MC- and phenobarbital-treated mice more so than that from control mice, but this difference was not significantly ($P > 0.05$) different statistically. The cytochrome *c* reductase activity was only slightly affected by these concentrations of metyrapone.

Three other commonly used Type II compounds—octylamine-1, pyridine and aniline—displayed (Fig. 6) the same preferential inhibition of the P-450 reductase activity, compared with little or no effect on the cytochrome *c* reductase activity. We also examined butanol-1, a compound having pure Reverse Type I character [20]: 40 mM butanol-1 inhibited the P-450 reductase activity only 15.7 ± 7.9 per cent (mean ± standard error from three experiments), and the cytochrome *c* reductase activity remained 100 per cent of normal (data not illustrated).

DISCUSSION

The findings in this report are consistent with the following hypothesis (Fig. 7). Evidence now exists from several laboratories [21–24] that one electron is transferred anaerobically from the reductase to cytochrome P-450 before atmospheric oxygen can

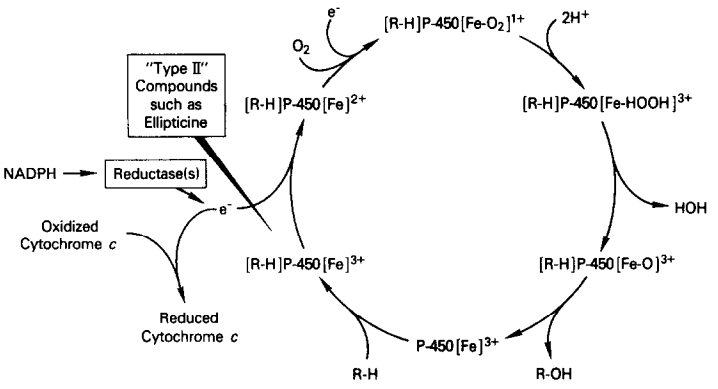

Fig. 7. Heuristic diagram of the reduction of P-450 ferric iron by electrons provided via one or more flavoprotein-containing reductases. After the entrance of one electron into the enzyme, molecular oxygen is able to bind, and the substrate (R-H) can receive the second electron and become oxygenated. The postulated point at which ellipticine and the other Type II compounds appear to interact is illustrated.

bind. Ellipticine (and other Type II compounds which bind with less avidity to P-450) thus appears to block the transfer of the first electron from the NADPH-reduced reductase(s). A very recent paper [25] indicates that electron transfer from the FAD to the FMN moiety of the reductase molecule is thermodynamically favorable. Therefore, the FMN is probably very close to, or may comprise, the site which binds to P-450 and passes the first electron to P-450. Whereas the electron transfer from the reductase to the P-450–substrate complex can be largely blocked by ellipticine and other Type II compounds, the electron transfer from the reductase to soluble cytochrome *c* remains relatively unaffected.

Trypsin treatment of the 76,000 dalton reductase molecule [24] yields an enzyme of about 69,000 daltons, which retains the hydrophilic binding site (capacity to reduce cytochrome *c*) yet loses its hydrophobic binding site (capacity to reduce P-450). In combination with that finding, the data in the present report suggest (Fig. 8) that the hydrophobic binding site of the reductase interacts favorably with the P-450–substrate complex in the presence of the Type I substrate or a Reverse Type I compound such as butanol. With the strong nitrogenous ligand of ellipticine and other Type II compounds, however, the P-450–substrate complex does not interact well with the hydrophobic binding site of the reductase. Whether the P-450 ferric iron is high spin (Type I) or 6-coordinated with a weak ligand (Reverse Type I) or 6-coordinated with a strong ligand (Type II) does not affect, moreover, the hydrophilic binding

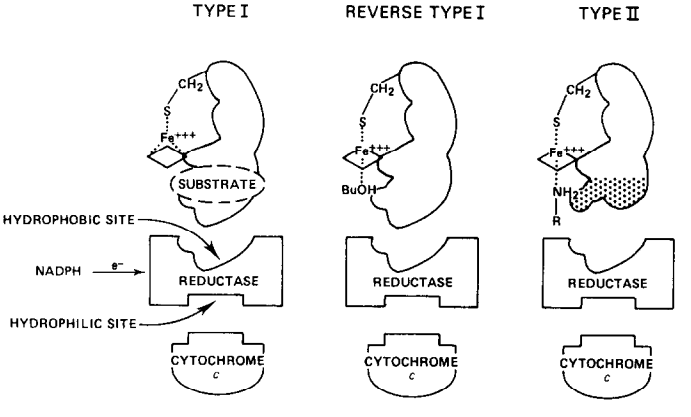

Fig. 8. Hypothetical diagram illustrating the hydrophobic (P-450 reductase) and the hydrophilic (cytochrome *c* reductase) binding sites on the flavoprotein reductase. High spin P-450 ferric iron with bound substrate (left) and low spin P-450 ferric iron coordinated with an oxygen in the sixth position as derived from butanol-1 (BuOH) (middle) are both able to bind and receive reducing equivalents from the reductase. Low spin P-450 ferric iron caused by Type II compounds ($R\text{-}NH_2$) (right), however, is unable to bind and receive in the usual manner the first electron from the reductase.

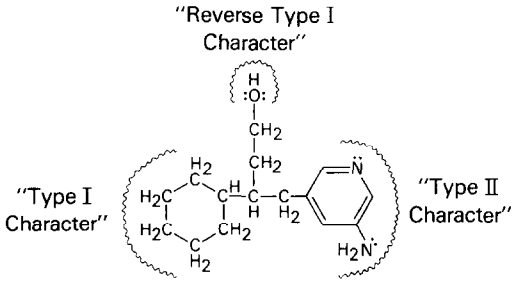

Fig. 9. Hypothetical chemical structure depicting a chemical which would interact with P-450 in three ways, producing three types of difference spectra due to "Type I", "Reverse Type I", and "Type II" character at various parts of the same molecule.

site of the reductase and its transfer of an electron to soluble cytochrome *c*. Previous reports [26,27] have indicated that Type II compounds cause a slower reduction of P-450 than do Type I compounds, but to our knowledge this is the first report which compares in the same liver preparation the effects of Type II compounds (and a Reverse Type I compound) on the P-450 reductase activity and the cytochrome *c* reductase activity.

Growing evidence implicates an ever increasing number of individual forms of P-450 [28–32]. "P-450 reductase activity" most likely represents the sum of numerous forms of P-450 being reduced at distinctly different rates.

It should be appreciated [20] that a single drug may have properties of "pure Type I character," "pure Reverse Type I character", and/or "pure Type II character" all within the same molecule (Fig. 9), and that each of these moieties may interact with the various forms of P-450 with varying K_s values. A barbiturate or a sex steroid is an example of a P-450 substrate having more than one type of binding within the same molecule; the difference spectrum of a sex steroid, for example, may therefore be Type I at low substrate concentrations and change to Reverse Type I at higher substrate concentrations. In this study we used octylamine-1, pyridine and aniline because they are compounds having predominantly Type II character; butanol-1 has relatively pure Reverse Type I character.

We have shown here that ellipticine binds to P-450 with an affinity greater than most other compounds known to interact with P-450 and that control, 3-MC-induced, and phenobarbital-induced mono-oxygenase activities known to be associated with numerous different forms of P-450 are all markedly inhibited by ellipticine to about the same extent. Metyrapone, for example, has an apparent K_s value of about 0.7 μM [33], yet preferentially binds to certain forms of P-450 other than P_1-450 [34] and preferentially inhibits mono-oxygenase activities associated with such forms of P-450. 1-(2-Isopropylphenyl)imidazole behaves similarly to metyrapone both in its high affinity for P-450 and in its preferential inhibition of the same types of mono-oxygenase activity [reviewed in Ref. 16]. α-Naphthoflavone preferentially binds to 3-MC-inducible forms of P-450 and preferentially blocks mono-oxygenase activities associated with such forms [35, reviewed in Ref. 16]. Why does ellipticine behave differently from such compounds as metyrapone, 1-(2-isopropylphenyl)imidazole, or α-naphthoflavone? Perhaps a second ellipticine molecule binds to the reductase in a site other than the sixth coordinate position which is shared with P-450 ferric iron. Further stoichiometric studies with purified reductase and purified forms of P-450 are indicated.

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