Pergamon

Biochemical Pharmacology, Vol. 48, No. 7, pp. 1421–1426, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00310-6

INHIBITION OF ADRENAL CYTOCHROMES P450 BY 1-AMINOBENZOTRIAZOLE *IN VITRO*

SELECTIVITY FOR XENOBIOTIC METABOLISM

DAN XU,* JEFFREY M. VOIGT,* BRUCE A. MICO,† SHIRO KOMINAMI,‡ SHIGEKI TAKEMORI‡ and HOWARD D. COLBY*§

*Department of Pharmacology and Toxicology, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104, U.S.A.; †Department of Drug Metabolism and Pharmacokinetics, Hoffmann–LaRoche, Inc., Nutley, NJ 07110, U.S.A.; and ‡Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima, 724 Japan

(*Received* 21 *March* 1994; *accepted* 26 *May* 1994)

Abstract—Studies were done to determine the effects of a P450 suicide inhibitor, 1-aminobenzotriazole (ABT), on adrenal steroid and xenobiotic metabolism. Incubation of guinea pig adrenal microsomes with ABT plus an NADPH-generating system caused a time-dependent decline in total P450 concentrations. The maximal decrease in P450 levels was approximately 35% and was accompanied by an equimolar decrease in heme content. Western blot analyses indicated that ABT had no effect on P450 apoprotein levels. Benzphetamine (BZ) *N*-demethylase and benzo[*a*]pyrene (BP) hydroxylase activities were inhibited almost completely by microsomal incubations with ABT. In contrast, neither steroid 17α-hydroxylase nor 21-hydroxylase activity was affected by ABT. The steroid-induced type I spectral change in adrenal microsomes also was not affected by ABT, whereas that induced by BZ was eliminated. Similar studies with adrenal mitochondria indicated that ABT had no effect on mitochondrial P450 concentrations or on mitochondrial steroid metabolism. The results demonstrate that the *in vitro* actions of ABT on adrenal cytochromes P450 are highly selective for those isozymes that catalyze xenobiotic metabolism. Therefore, ABT should serve as a useful probe for further characterization of adrenal xenobiotic-metabolizing P450 isozymes.

Key words: 1-aminobenzotriazole; cytochromes P450; adrenal cortex; xenobiotic metabolism; steroidogenesis

The adrenal cortex is an organ that contains an abundance of cytochromes P450 [1, 2]. Most adrenal P450 isozymes are involved in the synthesis of steroid hormones such as cortisol and aldosterone. However, foreign compounds are also metabolized by the adrenal cortex, mostly by P450 isozymes that are distinct from those involved in steroidogenesis [3, 4]. In addition, some xenobiotic-metabolizing activity has been attributed to the steroid hydroxylase P450c17 [5, 6]. The rates of xenobiotic metabolism are particularly high in the human fetal and guinea pig adrenal glands [7–9].

Although steroidogenic cytochromes P450 have been investigated extensively and are well-characterized [1, 2], relatively little is known about the adrenal isozymes that catalyze the metabolism of foreign compounds. It has been demonstrated that adrenal steroid- and xenobiotic-metabolizing activities are differentially regulated by a variety of physiological factors [7–9]. In addition, selective modulation of steroidogenic P450 isozymes by pharmacological agents has long been possible [10]. However, selective inhibitors of foreign compound metabolism by the adrenal have yet to be identified. The availability of such compounds would be very useful for studies on the further characterization of those adrenal P450 isozymes catalyzing xenobiotic metabolism.

ABT‖ is a mechanism-based (suicide) inhibitor of cytochromes P450 in various organs including liver, lung, and kidney [11–17]. Accordingly, inhibition by ABT requires metabolism by affected P450 isozymes in target organs and is time dependent; in addition, the effects are irreversible. Enzyme inactivation by ABT results from the alkylation of P450 heme by the ABT metabolite benzyne [11–13]. The inhibitory effects of ABT seem to be limited to cytochromes P450, but are relatively non-selective with respect to different P450 isozymes. However, effects on steroidogenic cytochromes P450 have not been investigated. A recent report indicating that adrenal enlargement results from ABT administration to rats [18] suggests the possibility of effects on adrenocortical function. The studies presented in this paper were done to pursue this observation by comparing the actions of ABT on adrenal P450-mediated steroid and xenobiotic metabolism.

MATERIALS AND METHODS

ABT was supplied by Hoffman–LaRoche, Inc.,

§ Corresponding author: Howard D. Colby, Ph.D., Department of Pharmacology and Toxicology, Philadelphia College of Pharmacy and Science, 600 South 43rd St., Philadelphia, PA 19104. Tel. (215) 596-8830; FAX (215) 895-1161.

‖ Abbreviations: ABT, 1-aminobenzotriazole; BZ, benzphetamine; and BP, benzo[*a*]pyrene.

Nutley, NJ. A polyclonal antibody to P4501A1 and P4501A2 that cross-reacts with a 52K isozyme in guinea pig adrenal microsomes was obtained from Human Biologics, Inc., Phoenix, AZ. A 3β-hydroxysteroid dehydrogenase inhibitor, 4,4-dimethyl-2α-cyano-20-spirox-5-en-3-one, was provided by Merck, Sharp & Dohme Research Laboratories, Rahway, NJ. Except where indicated, other reagents were obtained from the Sigma Chemical Co., St. Louis, MO.

Male English Short-Hair guinea pigs weighing approximately 800–1000 g were obtained from Camm Research Institute, Wayne, NJ. Animals were maintained under standardized conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°) and received food and water *ad lib*. All guinea pigs were killed by CO_2 inhalation between 8:00 and 9:00 a.m. Adrenal glands were quickly removed and placed in cold 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4) on ice. Adrenals were then trimmed free of fat and connective tissue, weighed, and homogenized in sucrose–Tris buffer. Washed mitochondrial and microsomal fractions were prepared by differential centrifugation as described previously [19, 20].

Incubation conditions for evaluation of the effects of ABT were essentially the same as described previously [20]. Microsomal suspensions (0.5 mg protein/mL) in 1.15% KCl–0.05 M Tris–HCl containing 3 mM $MgCl_2$ were incubated with ABT (0.1 mM) and/or an NADPH-generating system (0.4 mM $NADP^+$, 10 mM sodium isocitrate, 0.3 U/mL isocitrate dehydrogenase) in a total volume of 2.5 mL. Unless otherwise indicated, incubations were for 30 min in 25-mL Erlenmeyer flasks at 37° under air in a Dubnoff Metabolic Incubator. Mitochondrial incubations were done similarly except that 0.25 M sucrose was used in the medium instead of 1.15% KCl. For each of the incubation conditions employed, one set of flasks served as unincubated or 0-time controls. After the incubations, flasks were placed in an ice bath, and the mitochondria or microsomes in each flask were washed (recentrifuged) once prior to use in the assays described below.

Mitochondrial 11β-hydroxylase activity was assayed as the rate of conversion of 11-deoxycortisol to cortisol as described previously [19]. Cortisol was measured fluorometrically [21]. Cholesterol sidechain cleavage activity was determined as the rate of pregnenolone production by isolated adrenal mitochondria, with endogenous cholesterol as the substrate [19]. Cholesterol metabolism was initiated by the addition of 10 mM sodium isocitrate, and 4,4-dimethyl-2α-cyano-20-spirox-5-en-3-one (50 μM), a 3β-hydroxysteroid dehydrogenase inhibitor, was included in each flask to prevent the conversion of pregnenolone to progesterone [22]. Pregnenolone was extracted from the incubation flasks with methylene dichloride and measured with a highly specific radioimmunoassay [19].

Microsomal steroid 21-hydroxylase activity was determined from the rate of conversion of 17α-hydroxyprogesterone to 11-deoxycortisol, and 17α-hydroxylase activity from the rate of conversion of progesterone to 17α-hydroxyprogesterone plus 11-deoxycortisol. Incubation conditions and HPLC analyses of metabolites were described previously in detail [23]. Benzo[*a*]pyrene hydroxylation was determined by the fluorometric method of Nebert and Gelboin [24]. Quinine sulfate was calibrated against authentic 3-hydroxy-benzo[*a*]pyrene and routinely used as the fluorescence standard. Benzphetamine N-demethylation was assayed as the amount of formaldehyde formed using the method of Nash [25], as previously described [26]. For all enzyme assays, conditions were established to ensure linearity of product formation with respect to protein concentrations and incubation times.

Cytochrome P450 was measured as the dithionite-reduced CO complex as described by Omura and Sato [27]. Substrate-induced type I difference spectra were recorded with an Aminco DW-2a recording spectrophotometer in the split-beam mode. As noted above, all mitochondrial and microsomal preparations were reisolated (washed) after incubation with ABT and/or NADPH, and the spectra were obtained with the washed samples. Thus, most of the ABT and NADPH present during the incubations was removed prior to the spectral analyses. For western blot analyses, the anti-P450c17 and anti-P450c21 were prepared as described previously [28]. These antisera recognize adrenal microsomal proteins having molecular weights of 54K and 50K, respectively [3, 29]. A polyclonal antibody to P4501A1 and P4501A2 was used to monitor the 52K P450 isozyme in adrenal microsomes [3]. This antibody reacts with the microsomal protein having a molecular weight of 52K but not with the 54K (P450c17) or 50K (P450c21) protein. Similar observations have been made by Black *et al.* [3] using a different polyclonal antibody raised against rat P4501A1 and P4501A2. SDS–PAGE was done by the method of Laemmli [30] and western blots of microsomal proteins by the method of Towbin *et al.* [31]. Immunoreactive proteins were visualized by peroxidase immunostaining. Microsomal and mitochondrial protein concentrations were determined by the method of Lowry *et al.* [32]. Statistical analyses of differences between group means were done with the Newman–Keuls multiple-range test. Data are presented as means ± SEM.

RESULTS

Incubation of guinea pig adrenal microsomes with ABT plus an NADPH-generating system significantly decreased cytochrome P450 concentrations (Fig. 1). Neither ABT nor NADPH alone had any effect on P450 levels. These results are expected for a P450 suicide inhibitor and are similar to those obtained with ABT in liver and other organs [11–17]. Time-course studies revealed that the maximal loss of spectrally detectable P450 caused by ABT was 30–35% (Fig. 2). Higher concentrations of ABT did not increase the degradation of P450 (not shown). Thus, approximately 65–70% of adrenal microsomal P450 was resistant to the actions of ABT, a far greater fraction than reported for other organs [14–17]. Microsomal heme content was also decreased by ABT when NADPH was included in the incubation medium (Fig. 1). Although the percent decline in

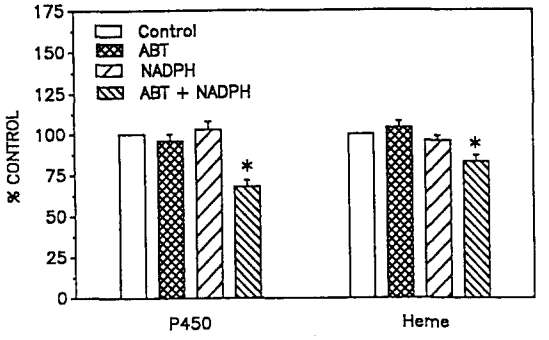

Fig. 1. Effects of incubating guinea pig adrenal microsomes with ABT (0.1 mM) and/or NADPH on P450 and heme concentrations. Incubations were done for 30 min as described in Materials and Methods. Data are expressed as percent of control values and are the means ± SEM of 4 experiments. Control values (100%): 1.9 ± 0.2 nmol/mg protein, P450; 4.1 ± 0.6 nmol/mg protein, heme. Key: (*) $P < 0.05$ (vs controls).

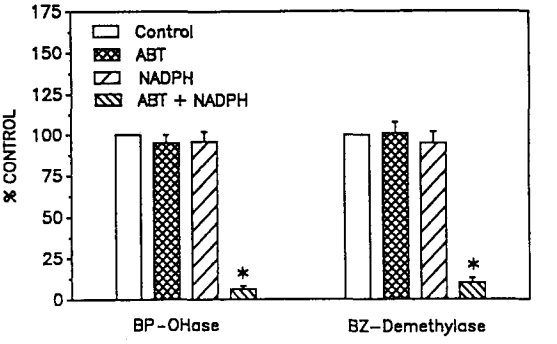

Fig. 3. Effects of incubating guinea pig adrenal microsomes with ABT (0.1 mM) and/or NADPH on xenobiotic metabolism. Data are expressed as percent of control values and are the means ± SEM of 5 experiments. Control values (100%): 467 ± 59 pmol/(min·mg protein), BP-OHase; 9.7 ± 1.4 nmol/(min·mg protein), BZ-demethylase. Key: (*) $P < 0.05$ (vs controls). Abbreviations: BP-OHase, benzo[*a*]pyrene hydroxylase; and BZ, benzphetamine.

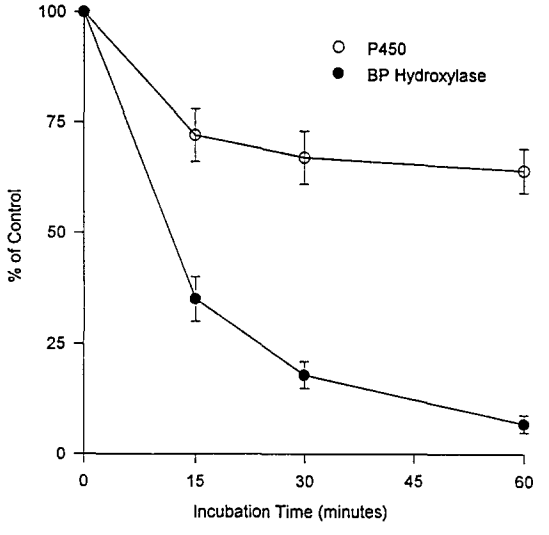

Fig. 2. Time–courses for the loss of cytochrome P450 and inactivation of BP hydroxylase during incubation of adrenal microsomes with ABT (0.1 mM) plus NADPH for 0, 15, 30 or 60 min. Incubations and assays were done as described in Materials and Methods. Values are means ± SEM of 4 experiments. Control values (100%): 1.8 ± 0.2 nmol/mg protein, P450; 516 ± 67 pmol/(min·mg protein), BP hydroxylase.

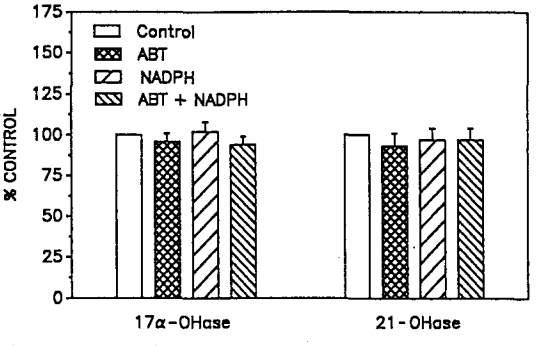

Fig. 4. Effects of incubating guinea pig adrenal microsomes with ABT (0.1 mM) and/or NADPH on steroid metabolism. Data are expressed as percent of control values and are the means ± SEM of 5 experiments. Control values (100%): 8.6 ± 1.1 nmol/(min·mg protein), 17α-OHase; 4.2 ± 0.6 nmol/(min·mg protein), 21-OHase. Abbreviations: 17α-OHase, steroid 17α-hydroxylase; and 21-OHase, steroid 21-hydroxylase.

total heme was smaller than that in P450, the molar losses were similar. The results suggest that ABT did not affect other hemeproteins such as cytochrome b_5, which is abundant in adrenal microsomes. ABT also had no effect on the concentrations of the apoproteins for the three major P450 isozymes in guinea pig adrenal microsomes: the P450c17 (54K), the P450c21 (50K), and the 52K isozyme that cross-reacts with antisera to P4501A1/P4501A2 [3]. Neither SDS–PAGE nor western blot analyses indicated any change in apoprotein levels (data not shown).

ABT had markedly different effects on adrenal xenobiotic and steroid metabolism. After incubation of microsomes with ABT plus NADPH, BP hydroxylase and BZ *N*-demethylase activities declined by more than 90% (Fig. 3). In the absence of NADPH, ABT had no effect on the rate of either reaction. Maximal loss of enzyme activity required incubation with ABT + NADPH for approximately 60 min (Fig. 2). In contrast to its effects on adrenal xenobiotic metabolism, ABT did not affect microsomal steroid hydroxylase activities (Fig. 4). The rates of 17α-hydroxylation and of 21-

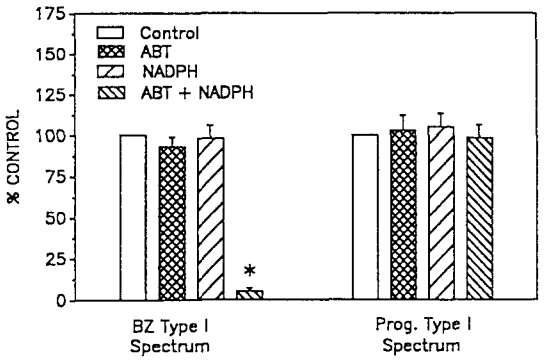

Fig. 5. Effects of incubating guinea pig adrenal microsomes with ABT (0.1 mM) and/or NADPH on the benzphetamine (BZ) and progesterone (Prog.)-induced type I difference spectra. Data are expressed as percent of control values and are the means ± SEM of 4 experiments. Control values (100%): 0.09 ± 0.02 (ΔA385 − 420 nm), BZ type I spectrum; 0.23 ± 0.03 (ΔA385 − 420 nm), prog. type I spectrum. Key: (*) $P < 0.05$ (vs controls).

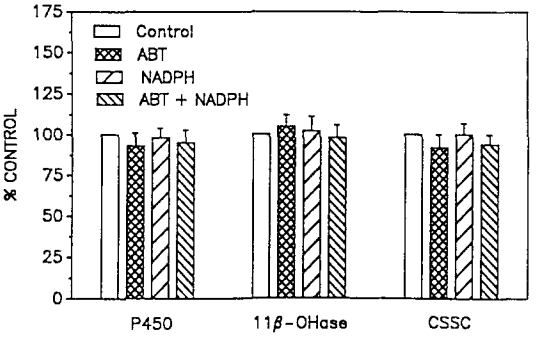

Fig. 6. Effects of incubating guinea pig adrenal mitochondria with ABT and/or NADPH on cytochrome P450 concentrations and on steroid 11β-hydroxylase (11β-OHase) and cholesterol sidechain cleavage (CSSC) activities. Data are expressed as percent of control values and are the means ± SEM of 5 or 6 experiments. Control values (100%): 0.7 ± 0.1 nmol/mg protein, P-450; 1.4 ± 0.2 nmol/(min·mg protein), 11β-OHase; 0.12 ± 0.02 nmol/(min·mg protein), CSSC.

hydroxylation were not altered by microsomal incubations with ABT in the presence or absence of NADPH. These findings suggested that the actions of ABT *in vitro* were limited to the xenobiotic-metabolizing P450 isozymes.

Spectral analyses of ABT effects on substrate-binding to microsomal cytochromes P450 yielded results that were consistent with the changes in enzyme activities. Binding of BZ to P450, as indicated by the magnitude of the type I difference spectrum, was eliminated almost completely by incubation of microsomes with ABT plus NADPH (Fig. 5). In contrast, the binding of progesterone, a steroid substrate for the 17α- and 21-hydroxylases, was not affected by ABT. These observations are consistent with a selective inactivation by ABT of adrenal microsomal P450 isozymes involved in xenobiotic metabolism, but not those catalyzing steroid synthesis.

Since some P450 isozymes that are involved in steroidogenesis are found in the mitochondrial fraction of adrenocortical cells, experiments similar to those described above were also done with mitochondrial preparations (Fig. 6). ABT had no effect on mitochondrial P450 concentrations or on the activities of the mitochondrial enzymes, steroid 11β-hydroxylase and cholesterol sidechain cleavage. Similarly, when whole adrenal homogenates were incubated with ABT + NADPH and subcellular fractions subsequently isolated, ABT had no effect on mitochondrial steroid hydroxylase activities, but almost completely inhibited microsomal xenobiotic metabolism (data not shown). Thus, all of the data indicate that the *in vitro* effects of ABT on adrenal P450 isozymes are limited to those involved in xenobiotic metabolism.

DISCUSSION

The results of prior investigations indicated that ABT was a relatively non-selective inhibitor of cytochromes P450 in livers, kidneys, and lungs [11–17]. In those organs, ABT effected the degradation of approximately 70–90% of the total P450 content. The effects of ABT on various isozyme-specific enzyme activities similarly suggested inactivation of multiple P450 isozymes. However, in none of the previous studies was the effect of ABT on steroidogenic cytochromes P450 evaluated.

The data presented in this report demonstrate that the actions of ABT *in vitro* on adrenal cytochromes P450 are highly selective for those isozymes that catalyze xenobiotic metabolism. Mitochondrial cytochrome P450-catalyzed steroidogenic reactions were not affected by ABT and the total concentration of mitochondrial cytochromes P450, which are involved in steroid metabolism only, was not diminished by ABT. In contrast, ABT caused a 30–35% decline in microsomal P450 concentrations and approximately 90% losses of BP hydroxylase and BZ *N*-demethylase activities. Microsomal 17α-hydroxylase and 21-hydroxylase activities were unaffected. Thus, the effects of ABT on adrenal cytochromes P450 might be characterized as virtually "all or none" responses. Sensitive isozymes were almost totally inactivated, whereas those resistant to ABT were not affected at all. It is possible that the small amount of xenobiotic-metabolizing activity not inhibited by ABT is catalyzed by the 17α-hydroxylase. Prior investigations have demonstrated that the P450c17 may participate in xenobiotic as well as in steroid metabolism [5, 6].

The mechanism of action of ABT on adrenal P450 isozymes appears to be the same as that in other organs. The NADPH requirement and time-dependence for ABT inactivation of adrenal xenobiotic metabolism are consistent with a mechanism-based (suicide) inhibition. In addition, the loss of microsomal heme, but not of P450 apoproteins, suggests that heme alkylation by an

ABT metabolite occurs. It is possible that apoprotein modification also occurred but without loss of immunoreactivity and, therefore, could not be detected by western blot analyses. The results of prior studies suggest that ABT can effect covalent binding to microsomal protein as well as to heme [14]. The ABT-induced decline in BZ binding to adrenal P450 noted in our experiments could be the result of direct effects at or near the substrate-binding site of the apoprotein or secondary to heme alkylation. Further investigation is needed to more fully define the mechanism of action. Because of the high selectivity of ABT for some adrenal cytochromes P450, further adrenal studies might also contribute information not readily obtained from other organs. In particular, the adrenal cortex might serve as a good model to investigate the structural requirements of P450 isozymes for the activation of ABT.

The maximal loss of cytochrome P450 caused by ABT in adrenal microsomes was approximately one-third of the total, as determined by the carbon monoxide difference spectrum, suggesting that this fraction corresponds to the major isozyme(s) involved in xenobiotic metabolism. However, definitive identification of this (these) ABT-sensitive isozyme(s) is not possible at this time. Adrenal steroidogenic cytochromes P450, both microsomal and mitochondrial, have been well characterized [1, 2], but relatively little is known about the isozyme(s) that catalyzes xenobiotic metabolism. Black and coworkers have demonstrated that the presence of a 52K protein is highly correlated with xenobiotic-metabolizing activity in guinea pig adrenal microsomes [3, 29], but this isozyme has yet to be purified or cloned. Band intensities on polyacrylamide gels indicate that the 52K isozyme comprises a substantial fraction of the total microsomal P450. The selectivity of ABT for xenobiotic metabolism in adrenal microsomes provides a potentially useful probe for further investigation on the isozyme(s) involved. Use of ABT should help in the identification of these isozymes as well as in the detailed characterization of their catalytic capabilities. Such studies are currently in progress.

Acknowledgements—These investigations were supported by Hoffmann–LaRoche, Inc. and by USPHS AG-11987.

REFERENCES

1. Hall PF, Cytochromes P-450 and the regulation of steroid synthesis. *Steroids* **48**: 131–196, 1986.
2. Takemori S and Kominami S, Adrenal microsomal cytochrome P-450 dependent reactions in steroidogenesis and biochemical properties of the enzymes involved therein. In: *Molecular Mechanisms of Adrenal Steroidogenesis and Aspects of Regulation and Application: Frontiers in Biotransformation* (Eds. Ruckpaul K and Rein H), Vol. 3, pp. 153–203. Akademie-Verlag, Berlin, 1990.
3. Black VH, Barilla JR, Russo JJ and Martin KO, A cytochrome P450 immunochemically related to P-$450_{c,d}$(P450I) localized to the smooth microsomes and inner zone of the guinea pig adrenal. *Endocrinology* **124**: 2480–2493, 1989.
4. Otto S, Marcus C, Pidgeon C and Jefcoate C, A novel adrenocorticotropin-inducible cytochrome P-450 from rat adrenal microsomes catalyzes polycyclic aromatic hydrocarbon metabolism. *Endocrinology* **129**: 970–982, 1991.
5. Suhara K, Fujimura Y, Shiroo M and Katagiri M, Multiple catalytic properties of the purified and reconstituted cytochrome P-450 (P-450_{sccII}) system of pig testis microsomes. *J Biol Chem* **259**: 8729–8736, 1984.
6. Mochizuki H, Kominami S and Takemori S, Examination of differences between benzo[*a*]pyrene and steroid hydroxylases in guinea pig adrenal microsomes. *Biochim Biophys Acta* **964**: 83–89, 1988.
7. Colby HD and Rumbaugh RC, Adrenal drug metabolism. In: *Extrahepatic Metabolism of Drugs and Other Compounds* (Ed. Gram TE), pp. 239–266. Spectrum Publications, Jamaica, NY, 1980.
8. Colby HD and Eacho PI, Chemical-induced adrenal injury: Role of metabolic activation. In: *Endocrine Toxicology* (Eds. Thomas JA, Korach KS and McLachlan JA), pp. 35–66. Raven Press, New York, 1985.
9. Hallberg E, Metabolism and toxicity of xenobiotics in the adrenal cortex, with particular reference to 7,12-dimethylbenz(a)anthracene. *J Biochem Toxicol* **5**: 71–90, 1990.
10. Gower DB, Modifiers of steroid-hormone metabolism: A review of their biochemistry and clinical applications. *J Steroid Biochem* **5**: 501–523, 1974.
11. Ortiz de Montellano PR and Mathews JM, Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. *Biochem J* **195**: 761–764, 1981.
12. Ortiz de Montellano PR, Mico BA, Mathews JM, Kunze KL, Miwa GT and Lu AYH, Selective inactivation of cytochrome P-450 isozymes by suicide substrates. *Arch Biochem Biophys* **210**: 717–728, 1981.
13. Ortiz de Montellano PR, Mathews JM and Langry KC, Autocatalytic inactivation of cytochrome P-450 and chloroperoxidase by 1-aminobenzotriazole and other aryne precursors. *Tetrahedron* **40**: 511–519, 1984.
14. Mathews JM, Dostal LA and Bend JR, Inactivation of rabbit pulmonary cytochrome P-450 in microsomes and isolated perfused lungs by the suicide substrate 1-aminobenzotriazole. *J Pharmacol Exp Ther* **235**: 186–190, 1985.
15. Mico BA, Federowicz DA, Ripple MG and Kerns W, *In vivo* inhibition of oxidative drug metabolism by, and acute toxicity of, 1-aminobenzotriazole (ABT): A tool for biochemical toxicology. *Biochem Pharmacol* **37**: 2515–2519, 1988.
16. Woodcroft KJ, Szczepan EW, Knickle LC and Bend JR, Three *N*-aralkylated derivatives of 1-aminobenzotriazole as potent and isozyme-selective, mechanism-based inhibitors of guinea pig pulmonary cytochrome P-450 *in vitro*. *Drug Metab Dispos* **18**: 1031–1037, 1990.
17. Mugford CA, Mortillo M, Mico BA and Tarloff JB, 1-Aminobenzotriazole-induced destruction of hepatic and renal cytochromes P-450 in male Sprague–Dawley rats. *Fundam Appl Toxicol* **19**: 43–49, 1992.
18. Meschter CL, Mico BA, Mortillo M, Feldman D, Garland WA, Riley JA and Kaufman LS, A 13-week toxicologic and pathologic evaluation of prolonged cytochromes P450 inhibition by 1-aminobenzotriazole in male rats. *Fundam Appl Toxicol* **22**: 369–381, 1994.
19. Colby HD and Eacho PI, Mitochondrial steroid metabolism in the inner and outer zones of the guinea pig adrenal cortex. *J Steroid Biochem* **23**: 477–484, 1985.
20. Sherry JH, O'Donnell JP, Flowers L, LaCagnin LB and Colby HD, Metabolism of spironolactone by adrenocortical and hepatic microsomes: Relationship

to cytochrome P-450 destruction. *J Pharmacol Exp Ther* **236**: 675–680, 1986.
21. Mejer LE and Blanchard RC, Fluorometric determination of plasma 11-hydroxycorticosteroids. II. Studies on the specificity of the method. *Clin Chem* **19**: 718–724, 1973.
22. Begue RJ, Gustafsson JA and Goldman AS, New potent inhibitors of 3β-hydroxy-Δ^5-steroid oxidoreductase with short duration of action. *Endocrinology* **95**: 238–256, 1974.
23. Eacho PI and Colby HD, Differences in microsomal steroid metabolism between the inner and outer zones of the guinea pig adrenal cortex. *Endocrinology* **116**: 536–541, 1985.
24. Nebert DW and Gelboin HV, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. *J Biol Chem* **243**: 6242–6249, 1968.
25. Nash T, The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**: 416–421, 1953.
26. Pitrolo DA, Rumbaugh RC and Colby HD, Maturational changes in adrenal xenobiotic metabolism in male and female guinea pigs. *Drug Metab Dispos* **7**: 52–56, 1979.
27. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
28. Kominani S, Shinzawa K and Takemori S, Immunochemical studies on cytochrome P-450 in adrenal microsomes. *Biochim Biophys Acta* **755**: 163–169, 1983.
29. Black VH, Immunodetectable cytochromes P450I, II, and III in guinea pig adrenal-hormone responsiveness and relationship to capacity for xenobiotic metabolism. *Endocrinology* **127**: 1153–1159, 1990.
30. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
31. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4357, 1979.
32. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.