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CHEMICAL AND BIOCHEMICAL CHARACTERISTICS OF *O*-DEMETHYLATION OF CHLOROTRIANISENE IN THE RAT*

PETER C. RUENITZ† and MO M. TOLEDO
School of Pharmacy, University of Georgia, Athens, GA 30602, U.S.A.

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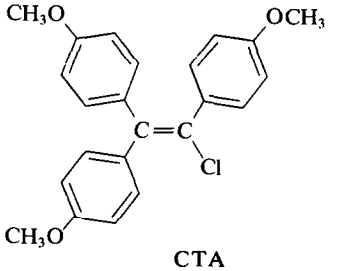
Abstract—The effects of NADPH concentration and of two inhibitors of the microsomal mixed function oxidase system [2-diethylaminoethyl-2,2-diphenyl valerate hydrochloride (SKF 525-A) and metyrapone] on rat liver microsomal *O*-demethylation of the triphenylethylene estrogen chlorotrianisene (CTA) were studied. Comparative data were obtained using untreated and phenobarbital-pretreated rats of both sexes. In the presence of microsomes from males, *O*-demethylation was induced slightly by phenobarbital (PB), and it was inhibited substantially by SKF 525-A, particularly with uninduced microsomes. Metyrapone had little inhibitory effect. In the presence of microsomes from females, *O*-demethylation was neither induced by PB nor inhibited significantly by SKF 525-A or metyrapone. Incubation of CTA with male rat microsomes afforded, after purification, a mixture of monophenolic metabolites which consisted primarily of a 1:1 mixture of *E*- and *Z*-desmethylchlorotrianisene (DMCTA).

Clinically useful nonsteroidal estrogenic activity has generally been associated with two structural categories [1]. These are the stilbene derivatives, of which diethylstilbestrol is best known, and the triphenylethylenes, best exemplified by chlorotrianisene (CTA). CTA is a long-acting estrogen which has been used in treatment of estrogen deficiency and prostate cancer and for suppression of postpartum breast engorgement in non-nursing mothers.



CTA

Characteristics of the estrogenic activity of CTA suggest that it undergoes metabolic activation by enzymes in the intestinal lumen, intestinal mucosa, and liver, since it is considerably more potent orally than parenterally. Since maximum potency is exhibited by related compounds (such as diethylstilbestrol) containing phenolic hydroxyl groups [1], the most logical pathway by which activation could occur would involve oxidative *O*-demethylation, resulting in the generation of a phenolic metabolite or metabolites. By analogy, the steroidal estrogen mestranol appears to owe its potent oral activity to its facile *O*-demethylation to ethinyl estradiol [2, 3].

We have shown that CTA undergoes *O*-demethylation to phenolic metabolites in the presence of rabbit liver microsomes [4]. Generation of these metabolites was dependent on the presence of NADPH, and was inhibited by carbon monoxide, indicative of the involvement of the microsomal mixed function oxidase system. We wanted to define more specifically the way in which CTA interacts with this system to undergo *O*-demethylation. Thus, in this paper we report the effects of cofactor concentration, and those of standard inhibitors and an inducer of the mixed function oxidase system, on the rate of *O*-demethylation of CTA in the rat. We also report the isolation and spectral characterization of the monophenolic metabolites of CTA, as produced in the presence of uninduced rat liver microsomes.

MATERIALS AND METHODS

Chemicals. 2-Diethylaminoethyl-2,2-diphenyl valerate hydrochloride (SKF 525-A) was obtained as a gift from Smith Kline & French Laboratories, Inc., Philadelphia, PA. The unambiguous synthesis of desmethylchlorotrianisene (DMCTA) will be described elsewhere. CTA and all other specialty chemicals and biochemicals used in this study were obtained from the Sigma Chemical Co., St. Louis, MO.

Preparation of microsomes. Male and female Sprague–Dawley rats were obtained from the Hiram–Davies Co., Stockbridge, GA. They were allowed unlimited food and water. Animals weighing about 150 g each were pretreated for 3 successive days with 80 mg/kg sodium phenobarbital (PB), given i.p. in normal saline (< 0.5 ml). Control animals received normal saline only. Animals were decapitated 24 hr after the last injection, and hepatic microsomal suspensions were prepared as reported previously [4].

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† Address correspondence to: Dr. Peter C. Ruenitz, School of Pharmacy, University of Georgia, Athens, GA 30602, U.S.A.

Incubation mixtures. Incubations were carried out in 25 ml Erlenmeyer flasks. The standard incubation mixture contained, in a volume of 5 ml, microsomal protein (10 mg), KCl (600 μmoles), potassium phosphate buffer, pH 7.4 (400 μmoles), and an NADPH-generating system consisting of NADP (2 μmoles), glucose-6-phosphate (40 μmoles), $MgCl_2$ (25 μmoles), and glucose-6-phosphate dehydrogenase (2.6 units). After a 3-min preincubation at 37°, 25 μl of 0.4 M CTA in acetone was added to give a final concentration of 2 mM, and the mixture was shaken for 20 min at 70 cycles/min in a metabolic shaker.

Assay for O-demethylation. Reactions were terminated and protein was precipitated as described previously [4]. The formaldehyde present was determined colorimetrically in aqueous solution by the procedure of Nash [5]. In experiments where increased sensitivity was necessary, aqueous solutions prepared by the Nash method were extracted with isoamyl alcohol and the clarified extracts were analyzed as reported by McLean and Day [6]. Incubation mixtures to which known amounts of formaldehyde had been added were simultaneously incubated and assayed as standards for quantitative analysis.

Thin-layer chromatography (t.l.c.). Analytical and preparative t.l.c. was carried out using Analtech 5 × 20 cm (0.25 mm thickness) and 20 × 20 cm (1 mm thickness) silica gel GF 254 plates respectively. Unless otherwise indicated, the solvent for t.l.c. was chloroform–methanol–28% aqueous ammonia (95:5:0.5, by vol.).

Detection and isolation of metabolites. Incubations were carried out for 30 min under standard conditions using twenty-four flasks, three of which did not contain the NADPH-generating system. The contents of these last three flasks were poured into a 45-ml centrifuge tube containing 15 ml of ethyl acetate and 1 g of NaCl (tube 1). The contents of the remaining flasks, in groups of three, were treated similarly (tubes 2–8). The eight tubes were shaken for 5 min and centrifuged at 1000 *g* for 15 min to clarify the upper phases. These phases, from tubes 2–8, were combined, concentrated, and compared by analytical t.l.c. with: (a) the concentrated ethyl acetate extract from tube 1; and (b) nicotinamide. The component, other than that with an R_f equal to that of nicotinamide, which was found only in the extract from incubation mixtures that contained NADPH, was subjected to purification from this extract residue by preparative t.l.c. (The concentrated extract was dissolved in methanol, and the resulting solution was extracted twice with *n*-hexane to remove endogenous lipids, prior to streaking it onto the preparative t.l.c. plate.) The appropriate silica gel band was removed and eluted with 5% (v/v) methanol in methylene chloride. Filtration and concentration of this extract gave the separated metabolite. Final purification was achieved by streaking a methanolic solution of this onto a 5 × 20 cm (0.25 mm thickness) plate, developing it with benzene–chloroform (50:50, v/v), and continuing as before. This gave a product that consisted of a single spot, R_f 0.61, as determined by analytical t.l.c. This was subjected to spectral analysis.

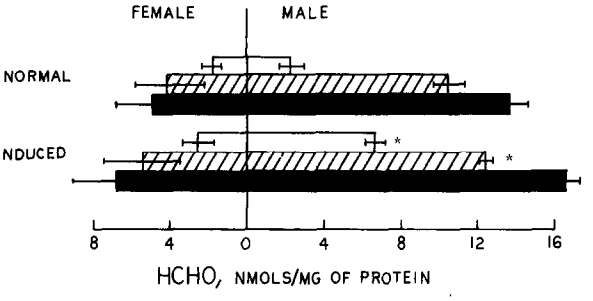


Fig. 1. Influence of NADPH on the comparative rates of *O*-demethylation of CTA in the presence of normal and phenobarbital-induced microsomes from male and female rats. NADPH concentration: (□) 0.2 mM, (▨) 0.4 mM, and (■) 0.8 mM. Standard deviations are indicated by brackets. Results are averages of three to five experiments with different animals. Asterisks (*) denote results from induced animals that differ significantly from those of normal animals ($P \leq 0.05$), using Student's *t*-test.

RESULTS

The effect of NADPH concentration on *O*-demethylation of CTA, as influenced by sex and pretreatment regimens of the rats used in liver microsomal preparations, is illustrated in Fig. 1. Formaldehyde production was greater in the presence of

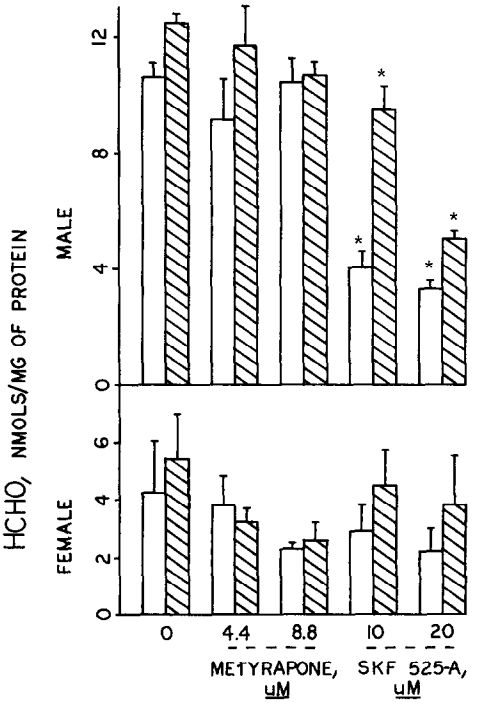


Fig. 2. Effects of metyrapone and SKF 525-A on the *O*-demethylation of CTA in liver microsomes from male and female rats pretreated with (□) saline, and (▨) phenobarbital. Standard deviations are indicated by brackets. Results are averages of three to five experiments with different animals. Asterisks (*) denote results that differ significantly from control values ($P \leq 0.05$), using Student's *t*-test.

Table 1. Diagnostic fragment ions in the mass spectra of DMCTA, CTA and its metabolite*

Compound	m/e (relative intensity) M+	M + 2	M −35
CTA	380 (100)	382 (40)	345 (40)
CTA metabolite	366 (100)	368 (52)	331 (52)
DMCTA	366 (100)	368 (42)	331 (50)

* Determined at 70 eV (probe temperature 220°) in a DuPont model 21-110 electron-ionization mass spectrometer.

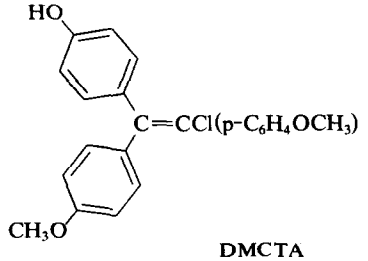
microsomes from untreated and phenobarbital-treated males than in the presence of those from similarly treated females. Microsomes from PB-treated females did not enhance formaldehyde production above that from controls, regardless of NADPH concentration. By contrast, microsomes from PB-treated males produced more formaldehyde than controls at all cofactor levels. The greatest enhancement was at a cofactor concentration of 0.2 mM.

Figure 2 summarizes the effects of SKF 525-A and metyrapone on O-demethylation of CTA in the presence of normal and PB-induced microsomes from male and female rats.

In the presence of induced microsomes from females, metyrapone caused a slight decrease in formaldehyde production, with a lesser (and non-significant) effect seen with normal microsomes. When incubations were carried out in the presence of SKF 525-A, no significant decrease in formaldehyde production was observed with either induced or normal microsomes.

In the presence of normal or induced microsomes from males, metyrapone had very little inhibitory effect. Marked inhibition was seen, however, in the presence of SKF 525-A, particularly with uninduced microsomes.

Analysis by t.l.c. of extracts from 30-min incubations of CTA with microsomes from untreated male rats indicated the presence of one major metabolite component (R_f 0.61) and two minor ones (R_f 0.16 and 0.23). Although these last two components were not present in sufficient quantities for isolation, the first one was. This product was chromatographically indistinguishable from authentic desmethylchlorotrianisene (DMCTA), and it had similar diagnostic mass spectral features (Table 1). The structure of this product was further confirmed by proton nuclear magnetic resonance (^{1}H n.m.r.)



DMCTA

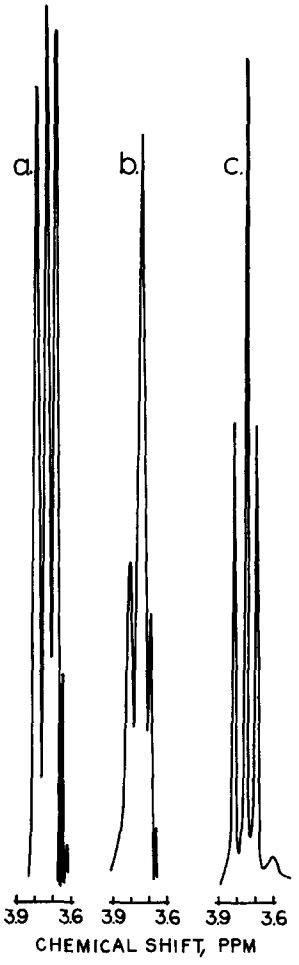


Fig. 3. Appearance of the OCH_3 signals (3.70 to 3.80 ppm) in the ^{1}H n.m.r. spectrum of (a) CTA, (b) authentic DMCTA, and (c) the microsomal metabolite of CTA. Data in (c) are from a 400 MHz spectrum recorded on a Varian XL 100 FT NMR spectrometer at the South Carolina Magnetic Resonance Laboratory (supported by NSF Grant CHE 78-18723). Data in (a) and (b) are from 60 MHz spectra obtained on a Hitachi R 20A spectrometer. All spectra were recorded using $CDCl_3$ as solvent and tetramethylsilane as internal standard.

spectral comparison with the authentic sample (Fig. 3). The chemical shifts of the OCH_3 protons in DMCTA and the metabolite(s) were identical.

DISCUSSION

Under the conditions of this study, CTA underwent O-demethylation to yield, primarily, a mono-O-demethylated product, as shown by t.l.c. and mass spectral comparison of this with authentic DMCTA.

Comparison of the ^{1}H n.m.r. spectrum of CTA with those of authentic and metabolic DMCTA gave further information regarding the structure of the monophenolic metabolite. As shown in Fig. 3a, CTA has three chemically nonequivalent OCH_3 groups. Comparisons of these data with analogous spectral data from closely related compounds allows assignment of these singlets to the respective OCH_3 groups

[7]. The singlets at 3.70 and 3.80 ppm are due to the anisyl methyl groups *trans* and *cis*, respectively, to the chlorine substituent. The remaining signal at 3.74 ppm is due to the methyl of the anisyl moiety which is geminal to the chloro substituent. Fortunately, the position of this signal remains constant when either one of the other two methyl groups is removed. Thus, in DMCTA (Fig. 3b) these protons appear as a singlet at 3.73 ppm. with the relative intensities of the singlets at 3.70 and 3.80 representative of the respective amounts of *E*- and *Z*-isomers* present. By this analysis, authentic DMCTA consists of a slight excess of the *Z*-isomer, while the metabolite (Fig. 3c) is comprised of approximately equal amounts of each isomer. Very little, if any, of the metabolite resulting from *O*-demethylation involving the anisyl moiety geminal to the chloro substituent is present. The apparent absence of this metabolite may be suggestive of an enzymatic preference for either of the geminal anisyl methyl groups of CTA. Alternatively, its absence could be a reflection of its chemical instability in incubation mixtures or during chromatographic purification.

Generation of phenolic metabolites from CTA in the presence of rabbit liver microsomes was found previously to require oxygen and NADPH, and was inhibited by carbon monoxide [4]. These requirements are, in general, similar to those for *O*-dealkylation of substrates by the cytochrome P-450-dependent mono-oxygenase system. The ability of this system to oxidize a particular substrate is also influenced differentially by inducers such as PB and 3-methylcholanthrene and by inhibitors such as SKF 525-A and metyrapone. This ability varies similarly with respect to species and sex differences.

In the presence of hepatic microsomes from male rabbits, CTA underwent *O*-demethylation at an apparent rate of 21.6 nmoles · (mg protein)$^{-1}$ · 20 min^{-1} [4]. This is approximately twice the rate seen in the presence of microsomes from male rats under identical conditions (Fig. 1).

As shown in Figs. 1 and 2, *O*-demethylation was faster in male rats than in females, the ratio in untreated animals being 2.6 (0.4 mM NADPH). Qualitatively similar results have been found in the *O*-demethylation of the steroidal estrogen mestranol and of *p*-nitroanisole, where under similar conditions the ratios were 1.36 and 1.66 respectively [8, 9]. Such differences have been ascribed to higher substrate binding capacity of cytochrome P-450 in male rats [10].

PB pretreatment caused an 18 per cent increase in formaldehyde production over controls (0.4 mM NADPH) in male rats (Fig. 1). No increases, however, were seen under similar conditions when 3-methylcholanthrene was preadministered (data not shown). Similar effects were observed in the rat liver microsomal *O*-demethylation of a structurally similar substrate, 4-methoxy-β-chlorostyrene. With this substrate, 3-methylcholanthrene had no stimulatory effect, but pretreatment with PB caused an 82 per cent increase in formaldehyde production [11]. Thus, these substrates are similar in that their dealkylations are apparently not catalyzed by the 3-methylcholanthrene-induced form(s) of cytochrome P-450, in contrast to certain other *O*-dealkylations [12]. These substrates, however, differ quantitatively in the involvement of PB-inducible cytochrome(s) P-450. This form of the enzyme is also more important in the *O*-demethylation of mestranol with respect to CTA. With this substrate, PB pretreatment resulted in 82 and 111 per cent increases in activity in male and female rats respectively [8].

Studies with metyrapone further suggested the limited involvement of PB-inducible cytochrome P-450 in the *O*-demethylation of CTA. This compound blocks the mono-oxygenation of substrates of PB-induced cytochrome(s) P-450 [13–15]. Thus, enzyme activity that is substantially induced by PB is also subject to substantial metyrapone inhibition. Activity was inhibited only slightly (14 per cent) in the presence of PB-induced male rat microsomes at 8.8 μM metyrapone, with no inhibition seen at the lower inhibitor concentration (Fig. 2). Inhibition appeared to be somewhat more pronounced with induced female rat microsomes, but quantitation was made uncertain by the low activities accompanied by relatively large standard errors associated with these data.

Activity in the presence of induced and normal microsomes from males was very sensitive to inhibition by SKF 525-A, with the former being somewhat less so. At a concentration of 10 μM, *O*-demethylation was inhibited by 24 and 62 per cent respectively. The degree of inhibition with normal microsomes is similar to that seen in analogously studied *O*-dealkylations of other substrates of the cytochrome P-450 system. Thus, the *O*-deethylations of ethoxyresorufin and ethylmorphine were reported to be inhibited by 35 and 70 per cent, respectively, in the presence of 2.5 μM SKF 525-A [12, 16].

Our results suggest that, in rat liver, CTA undergoes *O*-demethylation which is dependent mainly on noninducible cytochrome P-450. This affords a mixture of monodesmethyl metabolites, which consists primarily of a 1:1 mixture of the *E*- and *Z*-isomers of DMCTA, as determined after purification. These metabolites may account, in part, for the oral effectiveness of CTA.

In the triphenylethylene estrogens, amplified potency has been observed upon addition of phenolic hydroxyl groups [17]. Thus, potency of triphenylchloroethylene was increased by a factor of about forty by *p*-hydroxyl substitution in either of its geminal phenyl rings.

The prolonged estrogenic effects resulting from oral administration of CTA have been attributed to the tendency of this drug to accumulate in body fat [18, 19]. Results of the present study suggest that CTA may owe its prolonged activity not only to fat disposition, but also to its gradual *O*-demethylation resulting in the prolonged release of estrogenically active phenols.

* For a description of the use of affixes *E* and *Z* to indicate configurations of geometric isomers, see J. E. Banks, *Naming Organic Compounds*, 2nd Edn, pp. 73–9. Saunders, Philadelphia (1976).

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