[Chem. Pharm. Bull. 20(6)1145—1149(1972)]

UDC 547.92.09 : 615.31.015.4.076.9

Studies on Metabolism of 3-Deoxysteroids. X. Increased Activity of 3-Deoxyestrone Hydroxylases in Liver Microsomes of Rats pretreated with Phenobarbital and 3-Methylcholanthrene[1,2]

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(Received October 19, 1971)

Effects of the pretreatment with phenobarbital or 3-methylcholanthrene on the hydroxylase activity of rats liver microsomes were examined with 3-deoxyestrone. The administration of phenobarbital stimulated the 6β-hydroxylase, while the 3-methylcholanthrene treatment increased the rate of aromatic hydroxylation selectively. The biotransformation products formed from 3-deoxyestrone with the hepatic microsomes could be characterized by the reverse isotope dilution method and the chromatographic behaviors.

In the preceding papers of this series we reported the metabolic fate of 3-deoxyestrone, a lipid-shifting drug, in the rabbit.[4] With regard to the aromatic ring hydroxylation the occurrence of "NIH shift," which indicated the participation of the arene oxide as a possible intermediate, was observed.[5] The extensive studies in recent years have shown that the pretreatment with some drugs, polycyclic hydrocarbons or insecticides influences the activity of the steroid hydroxylases in the liver microsomes.[6] These investigations have so far been concerned with the enzyme systems which involve the hydroxylation of the endogenous steroid hormones and the biotransformation of the modified steroids has not hitherto been explored from this point of view. In this paper we wish to report the effects of the pretreatment of the rat with phenobarbital and 3-methylcholanthrene on the hepatic microsomal activity of 3-deoxyestrone hydroxylases.

Experimental

Animals——Male Wistar strain rats weighing 65 to 75 g were fed a synthetic diet. The rats were treated with intraperitoneal injections of phenobarbital (31.7 mg/kg) in saline (0.3 ml) twice daily for 4 days. 3-Methylcholanthrene (20 mg/kg) was injected intraperitoneally in sesame oil (0.25 ml) once daily for 5 days. Control rats received a similar intraperitoneal injection of the appropriate vehicles. All animals were starved for 24 hr prior to the sacrifice.

1) This paper constitutes Part LV of the series entitled "Analytical Chemical Studies on Steroids"; Part LIV: T. Nambara and Y.H. Bae, *J. Chromatog.*, **64**, 239 (1972).
2) The following trivial names are used: 3-deoxyestrone, estra-1,3,5(10)-trien-17-one; 3-deoxyestradiol, estra-1,3,5(10)-trien-17β-ol; estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol.
3) Location: *Aobayama, Sendai.*
4) T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 1200 (1969); *idem, ibid.*, **19**, 855, 990 (1971).
5) T. Nambara, M. Numazawa, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **19**, 153 (1971).
6) *a*) A.H. Conney and A. Klutch, *J. Biol. Chem.*, **238**, 1611 (1963); *b*) A.H. Conney and K. Schneidman, *J. Pharmacol. Exp. Ther.*, **146**, 225 (1964); *c*) R. Kuntzman, M. Jacobson, K. Schneidman, and A. H. Conney, *ibid.*, **146**, 280 (1964); *d*) T. Bledsoe, D.P. Island, R.L. Ney, and G.W. Liddle, *J. Clin. Endocrinol.*, **24**, 1303 (1964); *e*) S. Burstein and E.L. Klaiber, *ibid.*, **25**, 293 (1965); *f*) A.H. Conney, M. Jacobson, K. Schneidman, and R. Kuntzman, *Life Sci.*, **4**, 1091 (1965); *g*) R. Kuntzman, M. Jacobson, and A.H. Conney, *Pharmacologist*, **8**, 195 (1966); *h*) D. Kupfer and L. Peets, *Biochem. Pharmacol.*, **15**, 573 (1966); *i*) R.M. Welch, W. Levin, and A.H. Conney, *J. Pharmacol. Exp. Ther.*, **155**, 167 (1967); *j*) W. Levin, M. Ikeda, R. Kuntzman, D.Y. Cooper, and O. Rosenthal, *J. Biol. Chem.*, **243**, 3912 (1968).

Enzyme Assays——The rats were decapitated, livers from five animals were pooled, and a 10% liver homogenate was prepared in 0.25M sucrose solution at 0°. The nuclei and mitochondria were separated from the microsomes by centrifugation at $10000 \times g$ for 20 min. The microsomal pellet, which was obtained by centrifugation of the $10000 \times g$ supernatant fraction at $105000 \times g$ for 60 min, was washed twice with 0.25M sucrose solution and then suspended in ice-cold 1/30M KH_2PO_4–Na_2HPO_4 buffer (pH 7.4). For the enzyme assays a solution of 3-deoxyestrone-6,7-^{3}H (0.3 μmole, 0.95 μCi) dissolved in 95% EtOH (0.1 ml) was added to an ice-cold Erlenmeyer flask that contained NADP (4 μmole), glucose-6-phosphate (50 μmole), glucose-6-phosphate dehydrogenase (10 Kornberg Units), 0.05M $MgCl_2$ (0.25 ml), 0.05M tris(hydroxymethyl)-aminomethane buffer (pH 7.4) (0.25 ml) and a liver microsomal suspension equivalent to 400 mg of liver in a final volume of 5.35 ml. The mixture was incubated aerobically at 37° for 5 min. To the incubation mixture were added successively ether (20 ml) to stop the reaction and 2-hydroxy-3-deoxyestrone (400 μg) and estrone (400 μg) as a carrier. The mixture was extracted with ether (20 ml $\times$ 3) and partitioned with 2N NaOH (10 ml $\times$ 3). The radioactivity of the neutral metabolite obtained from the organic phase was assayed upon purification by thin-layer chromatography (TLC). The aqueous layer was adjusted to pH 1 with 6N HCl, saturated with NaCl and extracted with ether (20 ml $\times$ 3). After evaporation of the solvent, the residue was dissolved in MeOH (2 ml) and treated with 10% methanolic $NaBH_4$ solution (1 ml) under ice-cooling for 30 min. After decomposition of the excess reagent with a few drops of AcOH, the resulting solution was concentrated *in vacuo*. The residue thus obtained was submitted to column chromatography on Al_2O_3 as described below. The recovery rate of the inert carrier added was found to be *ca.* 69% when it was colorimetrically determined with conc. H_2SO_4.

Separation of Phenolic Metabolites by Column Chromatography——A solution of the phenolic metabolites dissolved in benzene (2 ml) was adsorbed on Al_2O_3 (E. Merck AG, activity II—III) (1.8 g) and then eluted with benzene (30 ml), 0.4% EtOH/benzene (60 ml) and 2% EtOH/benzene (60 ml), stepwisely. Each 2 g of the effluent was fractionally collected and evaporated *in vacuo* to give the residue, which in turn was dissolved in 99% EtOH (1 ml). A 0.2 ml aliquot was assayed for the radioactivity and the remainder was submitted to the colorimetric determination with use of conc. H_2SO_4. Hereupon, fr. 20—27 and 37—42 gave estradiol and 2-hydroxy-3-deoxyestradiol, respectively.

Thin-Layer Chromatography——Silica gel G (E. Merck AG) was employed as an adsorbent and activated at 110° for 90 min according to the Stahl's procedure. Hexane–AcOEt (2: 1) was used as a developing solvent unless otherwise specified. After development the adsorbent on the plate divided into each 0.5 cm width was eluted with acetone, and the eluate was assayed for the radioactivity.

Colorimetric Determination——Each fraction was evaporated to dryness *in vacuo*. To the residue obtained was added conc. H_2SO_4 (5 ml) and heated on a boiling water bath at 100° for 5 min. After cooling under the tap-water, the absorbance of the colored solution produced was read at 422 mμ for estradiol or at 372 mμ for 2-hydroxy-3-deoxyestradiol. The coloration obeys the Beer's law in the range of 10 to 150 μg of estrogen.

Synthesis of 3-Deoxyestrone-6,7-^{3}H——Estrone-6,7-^{3}H (The Radiochemical Centre, England, 500 mCi/mmole) (100 μCi) was diluted with nonlabeled estrone (5 mg) and transforemd into the 3-(1-phenyl-5-tetrazolyl)ether by condensation with 1-phenyl-5-chlorotetrazole in the presence of K_2CO_3. Hydrogenation over 5% Pd/$BaCO_3$ in benzene under a stream of H_2 gas gave 3-deoxyestrone-6,7-^{3}H (55 μCi/mmole).[5] The radiochemical purity was greater than 98% when assayed by TLC (*Rf* 0.64 (benzene)).

Counting of Radioactivity——Samples containing ^{3}H were counted in Packard Tri-Carb Model 3380 liquid scintillation spectrometer. Toluene containing 2,5-diphenyloxazole (4 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (200 mg/l) was used as a scintillant.

Result

Identification of Metabolites formed from 3-Deoxyestrone with Liver Microsomes

The thin-layer chromatogram of the ethereal extract of the incubation mixture indicated that the polar metabolites were formed from 3-deoxyestrone as was illustrated in Fig. 1. This extract could be divided into two fractions, the neutral and phenolic metabolites, when partitioned with 2N NaOH. The neutral metabolites, which exhibited *Rf* values of 0.64 and 0.29 by TLC, were characterized to be 3-deoxyestradiol (I) and 6β-hydroxy-3-deoxyestrone (II) by the reverse isotope dilution method, respectively (see Table I). On the other hand the phenolic fraction showed two radioactive spots (*Rf* 0.60 and 0.32) on the thin-layer chromatogram. Of these two the diol fraction was further subjected to column chromatography on alumina. The radioactive substance was eluted at fr. 20—27 as a single peak, which was identified as estradiol (III). The eluate obtained from another spot exhibiting the monohydroxylic polarity on the thin-layer plate was reduced with sodium borohydride and then

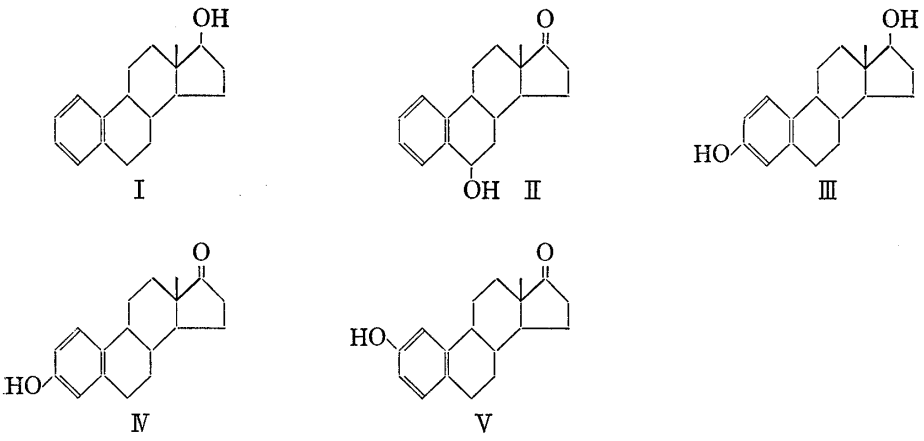

Chart 1. Metabolites Formed from 3-Deoxyestrone with Rat Liver Microsomes

chromatographed on alumina. Hereupon, the radioactive substances were separated into two peaks (fr. 20—27 and 37—42) and deduced to be estradiol and 2-hydroxy-3-deoxyestradiol on the basis of their chromatographic behaviors (see Fig. 2). The characterization of these reduction products could be confirmed by the reverse isotope dilution technique as listed in Table I. Thus the two phenolic metabolites were unambiguously assigned to the structures of estrone (IV) and 2-hydroxy-3-deoxyestrone (V).

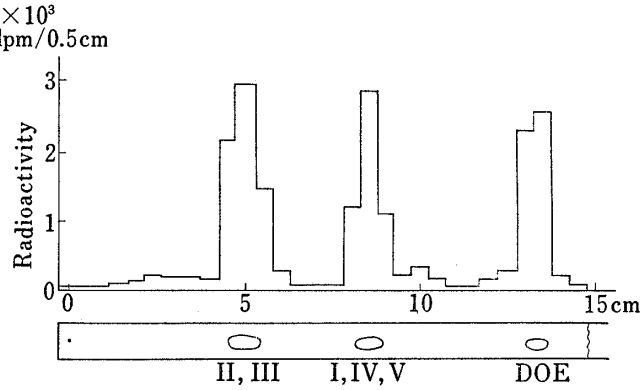

Fig. 1. Thin-Layer Chromatogram of Metabolites formed from 3-Deoxyestrone (DOE) with Rat Liver Microsomes

solvent: benzene-ether (2 : 1)
adsorbent: Silica gel G (E. Merck AG)

Table I. Identification of Metabolites Formed from 3-Deoxyestrone with Rat Liver Microsomes by Reverse Isotope Dilution Method

Compound	Recrystallization	Solvent	Specific activity (dpm/mg)
Estradiol[a]	1st	EtOH	8.76×10^3
	2nd	EtOH	8.75×10^3
	3rd	acetone	8.76×10^3
2-Hydroxy-3-deoxyestradiol[a]	1st	EtOH	6.55×10^3
	2nd	acetone	6.53×10^3
	3rd	EtOH	6.54×10^3
6β-Hydroxy-3-deoxyestrone[b]	1st	acetone	8.61×10^3
	2nd	acetone	8.23×10^3
	3rd	MeOH	8.15×10^3
	4th	acetone-H_2O	8.16×10^3
3-Deoxyestradiol[b]	1st	acetone–hexane	7.60×10^3
	2nd	MeOH	6.85×10^3
	3rd	MeOH	6.60×10^3
	4th	acetone–hexane	6.65×10^3

a) After the treatment with sodium borohydride followed by column chromatography on alumina, nonradioactive estradiol (20 mg) and 2-hydroxy-3-deoxyestradiol (35 mg) were added as a carrier, respectively.
b) Nonradioactive 6β-hydroxy-3-deoxyestrone (35 mg) and 3-deoxyestradiol (40 mg) were added as a carrier, respectively.

Effects of Pretreatment with Phenobarbital and 3-Methylcholanthrene on the Rate of Metabolism

The biotransformation rate of 3-deoxyestrone into the phenolic metabolites was not markedly influenced by the administration of phenobarbital. Furthermore, the ratio of the 2-hydroxylic metabolite to the 3-hydroxylic one was not altered by the liver microsomes of the drug-treated rats as was shown in Table II. In contrast it is evident from the data that the pretreatment with phenobarbital stimulated the transformation of 3-deoxyestrone into 6β-hydroxy-3-deoxyestrone and 3-deoxyestradiol by 200% and 170%, respectively. On

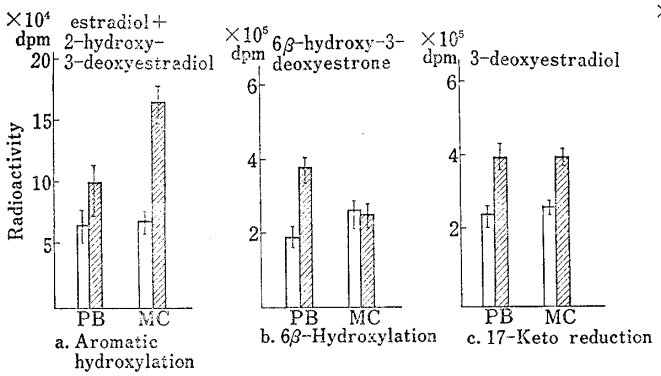

Fig. 3. Effects of Pretreatment with Phenobarbital and 3-Methylcholanthrene on Metabolism of 3-Deoxyestrone by Rat Liver Microsomes

□: control PB: phenobarbital-treated
▨: pretreatment MC: 3-methylcholanthrene-treated

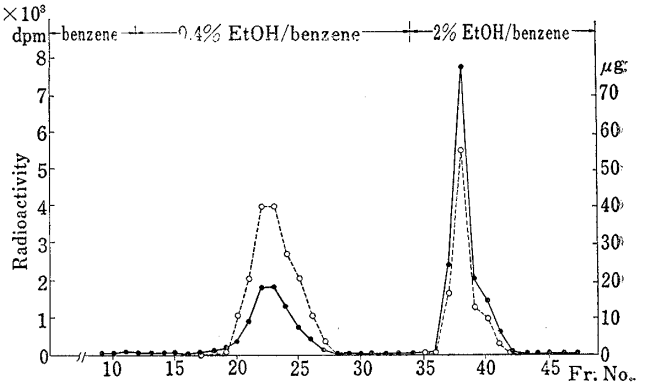

Fig. 2. Separation of Estradiol and 2-Hydroxy-3-deoxyestradiol by Column Chromatography

adsorbent: alumina (E. Merck AG), each fraction: 2g
—●—: radioactivity (^{3}H)
···○···: weight (by colorimetry)

the other hand, the intraperitoneal injection of 3-methylcholanthrene which is known as a selective inducer of certain drug-metabolizing enzyme, stimulated the conversion of 3-deoxyestrone into the phenolic metabolites by 240% and increased two fold the ratio of the 2-hydroxylic metabolite to the 3-hydroxylic one. The administration of this hydrocarbon, however, did not exert any influence on the 6β-hydroxylation and slightly stimulated the reduction of the 17-ketone into the 17β-hydroxy compound by 155% (see Fig. 3).

Table II. Effects of Pretreatment on the Relative Rate of 3-Deoxyestrone Aromatic Hydroxylation with Rat Liver Microsomes

Pretreatment	Ratio of hydroxylation rate (2-OH/3-OH)
Phenobarbital	3.56
Control	3.40
3-Methylcholanthrene	2.19
Control	1.05

Discussion

It has sufficiently been substantiated that there are many similarities between oxidative drug-metabolizing enzyme and steroid hydroxylase, and the oxidation of both steroids and drugs is catalyzed by the NADPH-dependent mixed function oxidase system localized in the liver microsomes.[7] These enzymes are well known to be induced by the pretreatment with some drugs, polycyclic hydrocarbons or insecticides. In addition, the stimulants are

7) A.H. Conney, *Pharmacol. Rev.*, **19**, 317 (1967); R. Kuntzman, *Ann. Rev. Pharmacol.*, **9**, 21 (1969).

divided into two groups, the nonspecific such as phenobarbital and the specific such as 3-methylcholanthrene.[7] The present study on 3-deoxyestrone demonstrates that the administration of phenobarbital stimulates the 6β-hydroxylase, whereas 3-methylcholanthrene does not exert any influence on the activity of this enzyme. This finding is in accord with the previous observation that the transformation rate of the endogenous steroid hormones, testosterone and androst-4-ene-3,17-dione, into the hydroxylated polar metabolites is significantly enhanced by the pretreatment of the rat with phenobarbital but not with 3-methylcholanthrene.[6a] In sharp contrast the 3-methylcholanthrene treatment selectively stimulates the aromatic A-ring hydroxylation and alters the relative activities of the two aromatic hydroxylases. This result appears to be analogous to the recent work reporting the enhanced hydroxylation rate for the aromatic compound after the treatment with the polycyclic hydrocarbon.[8] These results together imply that the separate enzyme systems catalyze the hydroxylation of 3-deoxyestrone in 2-, 3- and 6β-positions, respectively.

It has already been reported that the liver microsomal CO-binding pigment in animals treated with a polycyclic hydrocarbon such as 3-methylcholanthrene is different from the CO-binding pigment in control animals or in animals treated with phenobarbital with respect to the CO-difference spectrum.[9] The pretreatments with phenobarbital and 3-methylcholanthrene selectively induce the cytochromes P-450 and P-448, respectively, which have different catalytic activities.[9d] Other studies indicated that the enzyme activity localized in the rough surfaced and smooth surfaced microsomes is altered by the nature of the substrate, and the activity ratio of these two is also dependent upon the stimulant.[10] These facts seem to be attractive to explain our experimental result that the induction pattern is characteristic to each stimulant.

It is also of interest that the hydroxylation rate at C-2 relative to C-3 increases two fold after the administration of 3-methylcholanthrene. In our previous publication dealing with the "NIH shift" the arene oxide was assumed to be a possible intermediate leading to the 2- and 3-hydroxylated metabolites.[5] This explanation for the hydroxylation mechanism gives rise to a question whether the stimulatory pattern characteristic to 3-methylcholanthrene is due to the participation of other possible intermediate besides arene 2,3-oxide or to the alteration in the mode of ring opening for the common epoxide. It is hoped that further work in progress in this laboratory will provide the more precise knowlege on these problems.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.

8) D.W. Nehert and H.V. Gelboin, *J. Biol. Chem.*, **245**, 160 (1970).
9) *a*) A.P. Alvares, G. Schilling, W. Levin, and R. Kuntzman, *Biochem. Biophys. Res. Commun.*, **29**, 521 (1967); *b*) R. Kuntzman, W. Levin, M. Jacobson, and A.H. Conney, *Life Sci.*, **7**, 215 (1968); *c*) A. Hildebrandt, H. Remmer, and R.W. Estabrook, *Biochem. Biophys. Res. Commun.*, **30**, 107 (1968); *d*) A.Y.H. Lu, R. Kuntzman, S. West, and A.H. Conney, *ibid.*, **42**, 1200 (1971).
10) S. Orrenius and O.L. Ernster, *Biochem. Biohys. Res. Commun.*, **16**, 60 (1964).