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HEPATIC MICROSOMAL CONVERSION OF PREGNENOLONE TO 3β,5,6β-TRIHYDROXY-5α-PREGNAN-20-ONE VIA PREGNENOLONE α- AND β-EPOXIDES*

Tadashi Watabe, Mieko Kanai, Masakazu Isobe, and Naoki Ozawa

Laboratory of Drug Metabolism & Toxicology, Department of Hygienic Chemistry
Tokyo College of Pharmacy, Horinouchi, Hachioji-shi, Tokyo 192-03, Japan

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SUMMARY: In the presence of ferrous ion, ADP, and an NADPH-generating system, [4-^{14}C]pregnenolone was oxidized by bovine liver microsomes to its α-epoxide (5,6α-epoxy-3β-hydroxy-5α-pregnan-20-one), β-epoxide (5,6β-epoxy-3β-hydroxy-5β-pregnan-20-one), trihydroxypregnanone (3β,5,6β-trihydroxy-5β-pregnan-20-one) which were separated, isolated on an octadecylsilicone column in 70% aq. methanol by high performance liquid chromatography, identified with respective synthetic specimens by gas-liquid chromatography-mass spectrometry. The microsomal Δ^5-oxidation products of pregnenolone were detected in trace yield either when EDTA was added to the incubation mixture or when ferrous ion was omitted from the mixture. The microsomal oxidation system generated malondialdehyde significantly. It, however, was retarded to a negligible extent either by the addition of EDTA or by the omission of ferrous ion. Therefore, the microsomal formation of the significant yields of Δ^5-oxygenated pregnenolones was reasonably attributed to a reaction linked to microsomal lipid peroxidation. The ratio of pregnenolone α- to β-epoxides formed was 1:3. A comparable study carried out under the same conditions by using [4-^{14}C]cholesterol as the substrate resulted in the similar Δ^5-epoxidation with concomitant formation of cholestane-3β,5α,6β-triol; cholesterol α- and β-epoxides formed were in the ratio 1:4.

Both pregnenolone α- and β-epoxides were hydrolyzed by the microsomes to trihydroxypregnanone as the sole metabolite at a relative rate of 0.6:1. A similar relative value was also obtained in the microsomal hydrolysis of cholesterol α- and β-epoxides to the cholestanetriol.

INTRODUCTION

Hepatic microsomes oxidizes the cholesterol double bond in the presence of ferrous ion, ADP, and NADPH to yield cholesterol α-epoxide, cholesterol β-epoxide, and cholestanetriol (1). The α-epoxide has been demonstrated to cause chromosome aberration (2) and malignant transformations (3) of cells in culture, form covalent bindings to the bases of an isolated DNA (4) and be a possible causative material for hypercholesterolemic hyperpressure in man (5) as well as for skin carcinoma in hairless mice irradiated by ultraviolet ray (6). The fortifying agents, ferrous ion, ADP, and NADPH, are well known as the system

* Abbreviations used for trivial and systematic (in brakets) names of steroids are: pregnenolone (3β-hydroxy-5-pregnen-20-one), pregnenolone α-epoxide (5,6α-epoxy-3β-hydroxy-5α-pregnan-20-one), pregnenolone β-epoxide (5,6 β -epoxy-3β-hydroxy-5β-pregnan-20-one), trihydroxypregnanone (3β,5,6β-trihydroxy-5α-pregnan-20-one), cholesterol α-epoxide (5,6α-epoxy-5α-cholestan-3β-ol), cholesterol β-epoxide (5,6β-epoxy-5β-cholestan-3β-ol), and cholestanetriol (cholestane-3β, 5α,6β-triol).

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for microsomal lipid peroxidation, inducing a significant malondialdehyde formation from microsomes (7). Therefore, the microsomal cholesterol epoxidation was attributed to be a lipid peroxidation-mediated reaction (1). The lipid peroxidation-mediated cholesterol epoxidation also occurs in the lung of rats inhaled with nitrogen dioxide (8), in the bovine adrenal cortex mitochondrial preparation (9), in the system consisting of NADPH, NADPH-cytochrome *c* reductase, and an EDTA-ferrous ion (2:1) complex (10), in the soy bean lipoxygenase-linolate system (1), in the dispersion system containing cholesterol 7α-hydroperoxide or 6-cholesten-3β-ol 5α-hydroperoxide (11), and in organic solvents containing an acetylacetone-ferrous ion complex and *t*-butyl hydroperoxide or hydrogen peroxide (12).

The microsomal lipid peroxidation-mediated epoxidation has very recently been demonstrated by Kanai and Watabe (13) to occur not only with cholesterol, but also with other synthetic Δ^5-steroids, such as 5-cholestene and 20-methyl-5-pregnen-3β-ol, both of which yielded respective 5α,6α- and 5β,6β-epoxides (1:3-4) and 5α,6β-glycols. During the course of this investigation, the obligatory intermediacy of both stereoisomeric epoxides of these Δ^5-steroids to the corresponding 5α,6β-glycols was established by the isotope-trapping method, *i. e.* the conversion of the epoxides was catalyzed by microsomal epoxide hydratase. The enzymatic detoxication of the toxic epoxide, cholesterol α-epoxide, is also performed by hepatic soluble epoxide-S-glutathione transferase to afforded 5α-hydroxy-6β-S-glutathione conjugate (14).

The above mentioned facts regarding cholesterol and other synthetic Δ^5-steroids strongly suggest that lipid peroxidation of cell organella may epoxidize endogenous Δ^5-steroids other than cholesterol to reactive 5,6-epoxides. The present paper deals with the first evidence for the biotransformation of pregnenolone to trihydroxypregnanone *via* pregnenolone α-epoxide and pregnenolone β-epoxide by hepatic microsomes in the presence of NADPH and ferrous ion.

MATERIALS AND METHODS

Materials——NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Tokyo, unlabelled pregnenolone and pregnenolone acetate from Sigma Chemical Co., St. Louis, Mo., and unlabelled cholesterol, ADP, ferrous sulfate, and EDTA from Wako Pure Chemicals Co., Tokyo. [4-^{14}C]pregnenolone (55.7 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. and [4-^{14}C]cholesterol (58 mCi/mmol) from Radiochemical Centre, Amersham, England. The radioactive steroids were purified by HPLC before use. Pregnenolone β-epoxide (15) and trihydroxypregnanone (16), cholesterol α-epoxide (17), cholesterol β-epoxide (18), and cholestanetriol (16) were synthesized by the previously reported methods.

Pregnenolone α-epoxide that has been synthesized by several workers (16, 19, 20) by the methods accompanying with the formation of its β-epoxide isomer, was stereospecifically synthesized as follows: to a solution of pregnenolone acetate (1 g, 2.75 mM) in chloroform (10 ml) was added a solution of *m*-chloroperbenzoic acid (0.68 g, 3.93 mM, 85% purity as active oxygen) in chloroform (10 ml) during 30 min with stirring at room temperature. The reaction mixture

was left stand for 2 hr, shaken twice with an equal volume of 5% sodium carbonate, and then once with water. The residue obtained on the evaporation of the solvent from the chloroform solution was dissolved in methanol (20 ml) containing 10% sodium hydroxide (1.5 ml). The mixture was refluxed for 1 hr, diluted with ether (200 ml), shaken with water. From the etheral solution separated and dried over anhydrous sodium sulfate was evaporated the solvent, and the residue (1.1 g) obtained was recrystallized from acetone and isopropyl ether to give colorless needles (0.9 g, 90%), mp 181-182°. Nmr for pregnenolone α-epoxide δ (ppm from TMS in $CDCl_3$): 0.55 (18-H, 3H, *s*), 1.02 (19-H, 3H, *s*), 2.08 (21-H, 3H, *s*), 2.45 (17α-H, 1H, *t*, J 8 Hz), 2.90 (6β-H, 1H, *d*, J 6 Hz), and 3.80 (3α-H, 1H, *m*); for pregnenolone β-epoxide δ (ppm from TMS in $CDCl_3$): 0.56 (18-H, 3H, *s*), 0.96 (19-H, 3H, *s*), 2.07 (21-H, 3H, *s*), 2.45 (17α-H, 1H, *t*, J 8 Hz), 3.05 (6α-H, 1H, *d*, J 6 Hz), and 3.64 (3α-H, 1H, *m*).

Incubation Conditions——After the separation of the post-mitochondrial supernatant fraction obtained at 9000 x g for 20 min from a 3-volume homogenate of bovine liver taken at a slaughter house and immediately chilled in cold isotonic KCl solution, microsomes were isolated by the centrifugation of the supernatant at 105000 x g for 60 min. The microsomes were rehomogenized with 20 volumes of the KCl solution, recentrifuged under the same conditions, and suspended in 0.1 M phosphate buffer, pH 7.4, so that 1 ml of the suspension contained 13 mg protein determined by the method of Lowry *et al.* (21).

For the assay of microsomal oxidative metabolism of pregnenolone and cholesterol, a complete incubation system consisted of the microsomal suspension (1.3 ml, 17 mg protein), ^{14}C-pregnenolone (1 μCi, 2.4 μM) or ^{14}C-cholesterol (1 μCi, 24 μM) dissolved in acetone (100 μl), ferrous sulfate (0.2 mM), ADP (1.0 mM), NADP (1.0 mM), glucose 6-phosphate (10 mM), glucose 6-phosphate dehydrogenase (2 IU/ml), $MgCl_2$ (10 mM), and 0.1 M phosphate buffer, pH 7.4, to make a final volume of 8.5 ml. Prior to the biological reaction, the microsomal suspension was preincubated at 37° for 5 min with the radioactive substrate solution, and it was started by the addition of the other constituents.

For the assay of enzymatic hydrolysis of steroid epoxides, the microsomal suspension (13 mg protein/ml) was incubated in 0.1 M phosphate buffer, pH 7.4, with a solution of the substrate (0.1 mM) in acetone (2%). Both biological oxidation and hydrolysis reactions were terminated by the addition of an aqueous sodium hydroxide solution to make a final concentration of 1 M NaOH.

Isolation of Metabolites——Radioactive metabolites of [4-^{14}C]cholesterol or pregnenolone were extracted with ethyl acetate (20 ml) containing the corresponding unlabelled α-epoxide, β-epoxide, and 3β,5α, 6β-triol (1.0 mg each) as carriers. The residue obtained on the evaporation of the solvent was dissolved in benzene (5 ml) and poured onto a silica gel (5 g) column packed with benzene. For the removal of the unchanged substrate and non-polar lipids, column was eluted either with benzene and acetone (50:1) when ^{14}C-cholesterol was used as the substrate or with benzene and acetone (40:1) when ^{14}C-pregnenolone was as the substrate and then with benzene and acetone (1:1) for collecting all the 5,6-oxygenated metabolites in a fraction. From the chromatographic fraction containing radioactive metabolites and their carriers was evaporated the solvent, and the residue was dissolved in acetone and subjected to HPLC for the separation and isolation of the α-epoxide, β-epoxide, and triol.

Isolation of the triols yielded by microsomal hydrolysis of epoxides was carried out by the extraction of the alkalinized incubation mixtures with ethyl acetate (20 ml), followed by the application of the extract to HPLC.

Chromatography——HPLC (high performance liquid chromatography) was carried out on a Laboratory Data Control, Division of Milton Roy liquid chromatograph Model Constametric II G equipped with an octadecylsilicone column (Lichrosorb RP-18, 5 μ in particle size, 4 mm x 15 cm) and a Model 1107 Refracto Monitor. For the separation and isolation of cholesterol metabolites, the column was kept at 25° and developed with methanol at a flow rate of 1 ml/min. For pregnenolone metabolites, it was kept at 15° and developed with 70% aq. methanol at the same flow rate.

GLC (gas-liquid chromatography) was carried out on a Shimadzu Model GC-4CM equipped with a 5% SE-30 column (coated on 60-80 mesh Chromosorb W, 3 mm x 1 m) and a hydrogen flame ionization detector. For the assay of cholesterol metabolites, the column was kept at 280° and developed with nitrogen at a flow rate of 40 ml/min. For the pregnenolone metabolites, it was kept at 255° and developed at the same flow rate.

Radioactivity Counting——Radioactivities of the metabolites separated by HPLC were counted in a toluene scintillator on an Aloka Liquid Scintillation Counter Model LSC-502. The scintillator contained 0.03% dimethyl-POPOP and 0.5% DPO in toluene.

GLC-mass Spectrometry——GLC-mass spectra were recorded on a Shimadzu-LKB Model 9000 gas-chromatograph-mass spectrometer equipped with a 5% SE-30 (coated on 60-80 mesh Chromosorb W, 3 mm x 1 m). Column temperatures were 280° and 270° for cholesterol and pregnenolone metabolites, respectively. The flow rate of helium as the carrier gas was 30 ml/min. Other temperature conditions used were 290° for the injection port and the separator and 310° for the ion source chamber. The ionization energy applied was 20 eV.

RESULTS AND DISCUSSION

Isolation and Identification of Pregnenolone α- and β-Epoxides and Trihydroxypregnanone as Hepatic Microsomal Metabolites of Pregnenolone

[4-^{14}C]Pregnenolone was incubated at 37° for 40 min with bovine hepatic microsomes in the presence of an NADPH-generating system, ferrous sulfate, and ADP. The radioactive metabolites were extracted with ethyl acetate containing unlabelled pregnenolone α-epoxide, β-epoxide, and trihydroxypregnanone (1 mg each), as carriers. After the removal of the unchanged substrate and most of microsomal lipids from the extract on a silica gel column, the Δ^5-oxygenated steroid fraction obtained was subjected to HPLC for the separation and isolation of each steroid. In the high performance liquid chromatogram obtained on an octadecylsilicone column in 70% aq. methanol, pregnenolone α-epoxide, β-epoxide, and trihydroxypregnanone were well separated and showed retention times of 8.8, 7.2, and 4.0 min at 15°, respectively. An aliquot of the chromatographic fraction containing each steroid was used for the determination of radioactivities by the scintillation counting method and another aliquot for the determination of the amount of the carrier steroid by GLC: pregnenolone α-epoxide, β-epoxide, and trihydroxypregnanone had retention times of 7.5, 7.5, and 13.2 min, respectively, on a 5% SE-30 column at 255°. Specific radioactivities obtained from both data indicated that ^{14}C-pregnenolone was converted by the microsomal system to pregnenolone α-epoxide, β-epoxide, and trihydroxypregnanone in the ratio 1:3:1.5 (Table I).

Only very small amounts of radioactivities were found in the Δ^5-oxygenated pregnenolones when boiled microsomes were used or either when ferrous ion was scavenged by the addition of EDTA or when ferrous sulfate and ADP were omitted from the incubation mixture (Table I). Malondialdehyde formation determined by the thiobarbituric acid method (21) was significant in the complete incubation mixture and increased with linearity during the course of 60 min incubations

Table I. Lipid Peroxidation-mediated Δ^5-Oxygenation of Pregnenolone and Cholesterol in Bovine Hepatic Microsomes

	^{14}C-Products formed from (dpm/mg protein/min)[a]					
	Pregnenolone			Cholesterol		
	α-Epoxide	β-Epoxide	Triol	α-Epoxide	β-Epoxide	Triol
Complete[b]	10.7	29.2	15.2	9.4	42.1	6.5
Boiled microsomes used	n. s.	n. s.	n. s.	n. s.	n. s.	n. s.
Fe^{2+} and ADP omitted	n. s.	n. s.	n. s.	n. s.	n. s.	n. s.
EDTA added[c]	n. s.	n. s.	n. s.	n. s.	n. s.	n. s.

a n. s. represents radioactivities less than 0.9 dpm/mg protein/min.
b The complete system consisted of [4-^{14}C]pregnenolone or [4-^{14}C]cholesterol (1.0 μCi, 4 μM each), acetone (0.19 M), washed microsomes (13 mg protein/ml), NADP (1 mM), glucose 6-phosphate (10 mM), glucose 6-phosphate dehydrogenase (2 IU/ml), $MgCl_2$ (10 mM), $FeSO_4$ (0.2 mM), and ADP (1 mM).
c EDTA (1 mM) was added during preincubation of the substrate with the microsomes.

(1 nmol/mg microsomal protein/min). The malondialdehyde formation was retarded to a negligible extent by the use of boiled microsomes, by the addition of EDTA, or by the omission of ferrous sulfate and ADP. This strongly suggests the Δ^5-oxidation of pregnenolone to be linked to microsomal lipid peroxidation that is induced by ferrous ion and NADPH.

In order to obtain further evidence for the identification of the microsomal lipid peroxidation-mediated Δ^5-oxygenated products of pregnenolone, unlabelled pregnenolone (0.1 mM) was incubated with the complete system for 40 min. After the reaction was terminated, the mixture was extracted with ethyl acetate. The ethyl acetate extract was subjected to silica gel column chromatography and then to HPLC under the same conditions as used for the isolation of the radioactive metabolites. The separated HPLC fractions, corresponding to authentic Δ^5-oxygenated pregnenolone derivatives, proved by GLC-mass spectrometry to contain pregnenolone α-epoxide, β-epoxide, and trihydroxypregnanone. Their mass spectra (Fig. 1) and retention times in the chromatograms were identical with those of the authentic specimens. None of these Δ^5-oxygenated steroids was detected when boiled microsomes were used.

A comparable study of the microsomal Δ^5-oxidation of pregnenolone with that of cholesterol was made under the same conditions. The method used for the assay of radioactive metabolites of ^{14}C-cholesterol was substantially the same as used for that of pregnenolone metabolites and permitted the direct determination of cholesterol α-epoxide, β-epoxide, and cholestanetriol after the separation of them by HPLC; they had retention times of 8.2, 6.8, and 3.5 min, respectively, on the octadecylsilicone column in methanol at 25°. The ratio of α- to β-isomers of cholesterol epoxides was 1:4 (Table I), being in good accordance with

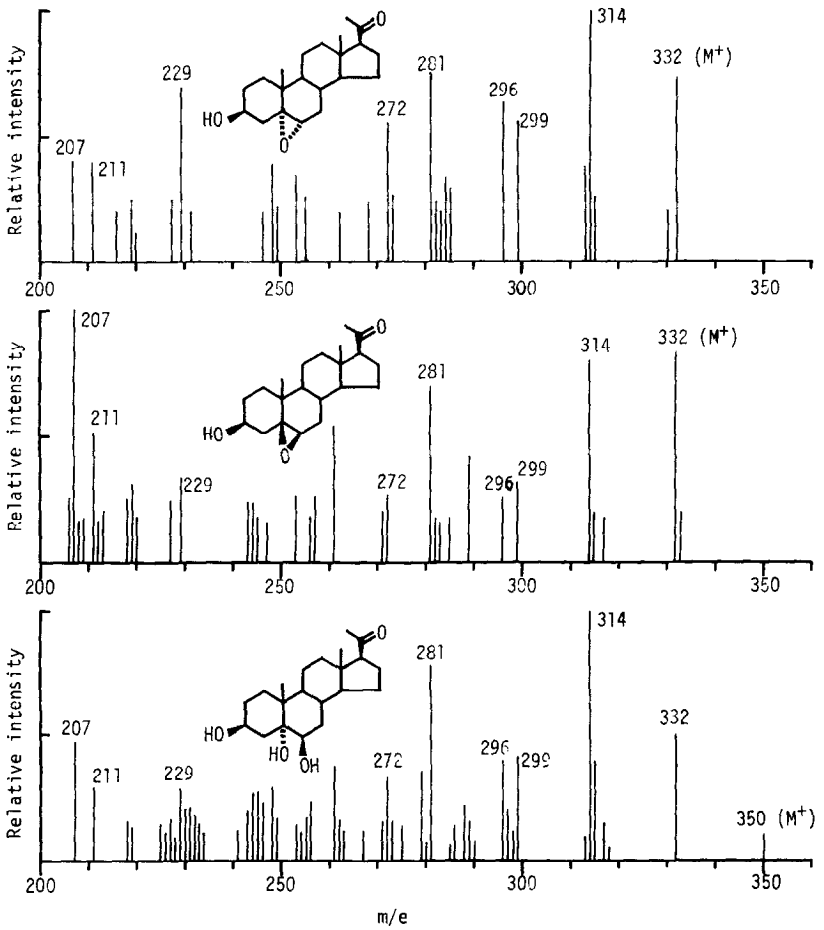

Fig. 1 GLC-mass spectra of Δ^5-oxygenated metabolites of pregnenolone by bovine hepatic microsomes in the presence of NADPH and ferrous ion-ADP complex. The metabolites were separated and isolated by HPLC. Analytical conditions are described in the text.

data obtained by Aringer and Eneroth (1) and by the other workers (12). A possibility of the participation of microsomal P-450 in the Δ^5-epoxidation of these steroids has been excluded by the fact that the microsomal epoxide formation is not influenced by carbon monoxide used as the gaseous phase in incubation mixtures (13).

Hepatic Microsomal Hydrolysis of Pregnenolone α-Epoxide and β-Epoxide to Trihydroxypregnanone

In order to confirm the microsomal enzyme activity which catalyzes the conversion of both biologically formed pregnenolone epoxides to trihydroxypregnanone, the unlabelled α- and β-epoxides were separately incubated with the hepatic microsomes in the phosphate buffer, pH 7.4, alone, and the polar trihydroxypregnanone fraction isolated on the octadecylsilicone column by

Table II. Hydrolysis of α- and β-Epoxides of Pregnenolone and Cholesterol to 3β,5α,6β-Triols by Bovine Hepatic Microsomal Epoxide Hydratase

Substrate (0.1 mM)	Triol formed[a] (pmoles/mg protein/min)	Relative rate of hydrolysis (α/β)
Pregnenolone		
α-epoxide	21.7	
β-epoxide	37.9	0.57
Cholesterol		
α-epoxide	48.0	
β-epoxide	69.4	0.69

a The triol formed from both pregnenolone α- and β-epoxides was 3β,5,6β-trihydroxy-5α-pregnan-20-one, and the one from cholesterol α- and β-epoxides was cholestane-3β,5α,6β-triol.

HPLC was subjected to GLC-mass spectrometer. The mass spectra obtained from both pregnenolone α- and β-epoxides as substrates were identical with those of the authentic specimens. Relative rate of microsomal hydrolysis of the α-epoxide to the β-epoxide was 0.6 (Table II). No trihydroxypregnanone formation was observed when the epoxides were incubated with boiled microsomes, indicating the hydrolytic reaction to be enzymatic.

A comparable study carried out with cholesterol α- and β-epoxides resulted in the similar relative rate (Table II). A reasonable interpretation of the preferential enzymatic hydrolysis of β-epoxides to that of the α-isomers would be attributable to a steric hindrance effect of their 10β-methyl groups on the introduction of the hydroxyl anion from water to their 6β-positions during the course of enzymatic hydrolysis of the α-epoxides which leads to the 5α,6β-dihydroxy steroids as a result of *trans*-diaxial fission (Fig. 2). Very similar results have been obtained by Watabe *et al.* (23) in the microsomal hydrolysis of 5α-2-cholestene α- and β-epoxides, the latter of which is hydrolyzed faster than the former (α/β=0.035). The smaller α/β ratio in enzymatic hydrolysis of

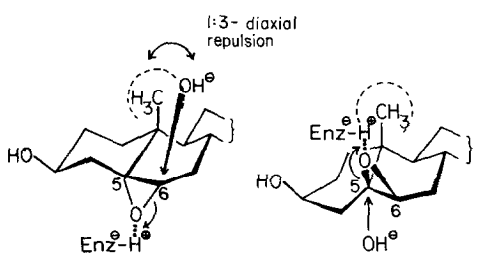

Fig. 2 Possible mechanisms for enzymatic hydrolysis of Δ^5-steroid α- and β-epoxides. Enz-H represents a proposed active center with a dissociating hydrogen with which the oxirane oxygen of epoxides interacts. In the β-epoxide molecules, the introduction of OH^- from water may be hindered by the steric effect of the 10-methyl group.

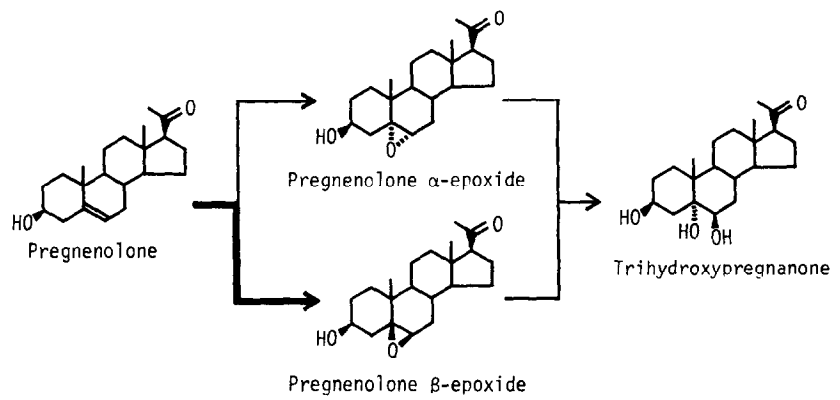

Fig. 3 Microsomal lipid peroxidation-mediated pregnenolone epoxidation and microsomal epoxide hydrolysis.

the Δ^2-steroid epoxides than that of Δ^5-steroid epoxides would be due to a hindered interaction of the hydroxyl anion on the fission of their oxirane rings with their 6α-positions which are located at the bottom of a cage structure of the β-epoxide molecules with A/B-*cis*-ring juncture.

Thus, a new oxidative metabolism of pregnenolone by bovine liver microsomes in the presence of the ferrous ion-ADP complex and NADPH has been established as illustrated in Fig. 3.

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