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# INVESTIGATION OF RAT LIVER MICROSOMAL 6β-HYDROXYLATION OF 4-ANDROSTENE-3,17-DIONE AND 4-PREGNENE-3,20-DIONE USING METHODOLOGY WHICH EXCLUDES STEROID-3-IMINE INDUCED INTRODUCTION OF THE 6β-HYDROXYL GROUP

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**Summary**—To avoid artefactual 6β-hydroxylation of 3-oxo-4-ene steroids due to steroid-3-imine formation and rearrangement a combined extraction and liquid chromatography purification procedure for incubated rat liver microsomes has been worked out. With this procedure no nonenzymatic 6β-hydroxylation could be observed. Conventional termination of incubations with male rat liver microsomes (105,000 *g* sediments) and 4-$^{14}$C-labelled 4-androstene-3,17-dione (or progesterone) by solvent extraction and evaporation might lead to a 30% overestimation of the formation of 6β-hydroxy-derivatives at substrate saturation. Furthermore this work-up procedure produces 6-oxo-derivatives which were not observed when the new procedure was used. By elimination of the artefactual 6-oxygenation some properties of the male rat liver microsomal 4-androstene-3,17-dione 6β-hydroxylase have been studied, and the activity has been compared to the artefact produced by solvent extraction and evaporation. Using the combined extraction–liquid chromatography purification it was demonstrated that the microsomal 6β-hydroxylase active on 4-androstene-3,17-dione and progesterone was inhibited to 95% by carbon monoxide. This makes previous suggestions regarding participation of a non cytochrome $P_{450}$-dependent 4-androstene-3,17-dione 6β-hydroxylase less likely.

## INTRODUCTION

The microsomal 6β-hydroxylation of 3-oxo-4-ene-steroids, e.g. testosterone, 4-androstene-3,17-dione and progesterone in the liver of rats and other species has received a lot of interest since the activity has been shown to be influenced by sex [1] as well as age [2], and to be induced by endogenous compounds [3] as well as xenobiotics [4]. Several cytochrome $P_{-450}$ species have been isolated with the ability to perform 6β-hydroxylation of 3-oxo-4-ene-steroids in reconstituted systems [5,6,7,8]. But recovery of only 10–20% of the total microsomal 6β-hydroxylation activity has been reported after purification of several cytochrome $P_{-450}$:s from rats treated with 3-methyl-cholantrene [5]. This and the fact that the known cytochrome $P_{-450}$ inhibitor carbon monooxide does not completely abolish the 6β-hydroxylation of testosterone [4] and progesterone [9] has lead to the speculation that other enzymes in liver microsomal preparations, such as the lipoxygenase(s) may participate in 6β-hydroxylation. Thus 6β-hydroperoxy-4-pregnene-3,20-dione and its decomposition products 6β-hydroxy-4-pregnene-3,20-dione and 4-pregnene-3,6,20-trione were identified after incubation of rat liver microsomes with progesterone [9]. Analogous results were obtained with 4-androstene-3,17-dione incubated with human placental microsomes [10]. In the two cited studies a high conversion of the steroid substrates to 6-oxygenated derivatives was noted even with boiled controls. Consequently the need for carefully conducted control experiments was stressed.

The problems experienced in studies on 6β-hydroxylation may be related to steroid-3-imine formation between the 3-oxo-4-ene-steroid and lipophilic amines during evaporation of the organic solvent extract of the incubated subcellular fractions. Thus we recently demonstrated the formation of 6β-hydroxy-, 6-oxo, and 6β-hydroperoxyderivatives during hydrolysis of 3-imines of 4-ene-3-one steroids [11]. This reaction sequence was also shown to take place in organic solvent extracts of rat liver subcellular fractions to an extent that might obscure measurements of the 6β-hydroxylase activity [12]. In the present paper we have investigated male rat liver microsomal steroid 6β-hydroxylase activity using a method by which artefactual steroid-imine formation is avoided.

## EXPERIMENTAL

### *Reagents*

All solvents and reagents were of analytical grade and purchased from E. Merck A. G., Darmstadt, West Germany, unless otherwise stated. They were used without further purification.

*Radioactive compounds*

[4-$^{14}$C]4-Androstene-3,17-dione (2.03–2.07 GBq), and [4-$^{14}$C]4-pregnene-3,20-dione (1.96–2.03 GBq) were purchased from the Radiochemical Centre, Amersham, England.

Unlabelled reference steroids were purchased from Steraloids Inc., NH, U.S.A., with the exception of 16α-hydroxy-4-pregnene-3,20-dione which was a gift from Professor J.-Å. Gustafsson, Huddinge Hospital, Stockholm, Sweden.

The purity of the steroids was checked by thin-layer chromatography (TLC), gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS) as described previously [11] (and below). All steroids were freshly purified on the day preceding the experiments.

*Cofactors and enzymes*

Glucose-6-phosphate-dehydrogenase type V, D-glucose-6-phosphate disodium salt; hydrate, Sigma grade, nicotinamide adenine dinucleotide phosphate (NADP) tetrasodium salt type III, were purchased from Sigma Chemical Company, St Louis, MO, U.S.A.

*Liquid chromatography* (LC)

Lipidex-1000, Packard Instrument Co., (IL, U.S.A.) was used in methanol–water–*n*-butanol–chloroform, 60:40:7:3 (by vol). Column dimensions: 0.75 cm$^2$ × 13 cm.

*Thin-layer chromatography* (TLC)

Precoated Silica Gel 60 plates (20 × 20 cm, Merck) were used in the solvent systems described in footnotes to Fig. 5 and Fig. 6 unless otherwise stated. The solvent was allowed to rise 20 cm once. Separated compounds were detected by iodine vapour or by radioautography. Compounds were extracted with 5 ml of methanol/cm$^2$ of gel followed by 5 ml chloroform–methanol, 1:1 (by vol)/cm$^2$ of gel.

*Gas–liquid chromatography* (GLC)

A Pye gas chromatograph (Model 104) equipped with a hydrogen flame ionization detector (FID) was used. The steroids were analyzed using a column (2 m × 4 mm) containing Supelcoport, 100–120 mesh, coated with 3% SE-30 (Supelco Inc. Bellefonte, PA, U.S.A.). Temperatures; flash heater, 270°C; column oven, 245°C; detector oven, 270°C. Carrier gas was nitrogen at a flow rate of 480 ml/cm$^2$/min. Relative retention times ($t_R$) were calculated using 5α-cholestane as reference.

*Gas chromatography–mass spectrometry (GC–MS)*

An LKB 9000 instrument equipped with a 1.5% SE-30 column (1.5 × 3 mm) or a Finnigan instrument model 1020 equipped with a 25 m fused silica SE-30 column were used. The LKB instrument used helium as carrier gas and the energy of bombarding electrons was 22.5 eV. Temperatures were: ion source, 290°C; flash heater, 270°C; column oven, 260°C; molecular separator, 280°C. The Finnigan instrument used helium as carrier gas (40 KPa) and the energy of bombarding electrons was 40 eV. The samples were dissolved in heptane and introduced split-less on the column which was temperature programmed between 190 to 260°C with an increase of 2°C/min. Molecular separator temperature was 280°C.

*Measurement of radioactivity*

A Packard 2009 liquid scintillation spectrometer was used. Six ml of scintillation cocktail (Insta Gel, Packard) was added to an aliquot (50–250 μl) of the sample. $^{14}$C-Labelled spots on TLC were located by exposing a sheet of Agfa Gevaert Osray M3 film to the plate for 1–14 days. GLC-radioactivity detection (GLC–RD) was accomplished with a Packard instrument 893.

*Animals*

Male Sprague–Dawley rats (280–300 g, ALAB, Stockholm, Sweden) were kept for 4–5 days preceding the experiments. They were fed a standard pellet food (ALAB) and water *ad libitum*. For each experiment, combined organs from 3–5 animals were used. The animals were sacrificed by a blow to the head at 8 a.m.

*Preparation of rat liver homogenates*

The livers were excised and rinsed in an ice-cold 0.25 M sucrose solution and the mixture was homogenized utilizing a Potter–Elvehjem homogenizer with a Teflon pestle (clearance 0.15 mm). All steps were carried out at 4°C.

*105 000 g sediments*

These were prepared at 4°C in a 0.1 M phosphate buffer, pH 7.4 with a NADPH regenerating system as described by Mode *et al.* [13]. Unless otherwise stated 0.5 ml (2–4 mg of protein, measured as described by Lowry *et al.* [14]) of the 20% w/v microsomal preparation was used and 0.5 ml of the same buffer containing 2.5 units of glucose-6-phosphate dehydrogenase, 5 μmol of glucose-6-phosphate and 1.5 μmol of NADP was added.

*Incubation and purification procedures*

The steroid substrate was added dissolved in 100 μl of acetone with concomitant vibration under a stream of nitrogen. The incubation mixture was placed in a water bath at 37°C and air was blown over the mixture to remove the nitrogen. Unless otherwise stated the mixture was incubated for 10 min. In experiments which were analyzed by a direct liquid chromatography step, incubations were terminated by adding 2.65 ml of methanol–*n*-butanol–chloroform, 60:7:3 (by vol). The mixture was centrifuged at

2000 *g* and the resulting supernatant was directly applied to a 0.75 $cm^2$ × 13 cm column of Lipidex-1000 in methanol–water–*n*-butanol–chloroform, 60:40:7:3 (by vol) and eluted in the same solvent system with a flow rate of 0.3 ml/$cm^2$/min. In experiments with 4-androstene-3,17-dione the fractions eluted between 115–200% of the total column volume (%TCV) were combined and evaporated prior to identification and quantitation (these fractions contained between 90–102% of the added $^{14}C$-activity, $n = 15$). In experiments with 4-pregnene-3,20-dione the fractions eluted between 140–300% TCV were collected prior to analysis (recoveries were 90–98%, $n = 10$). The remaining material on the column containing between 2–5% of the added radioactivity was eluted by 10 ml of acetone. This fraction contained mainly the 5α-reduced metabolites of progesterone, 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one but also some 2α-hydroxy-4-pregnene-3,20-dione and 20α- and 20β-hydroxy-4-pregnen-3-one (GC–MS). No 6β-hydroxy-4-pregnene-3,20-dione could be detected in this fraction.

In order to check for specific steroid losses in this procedure the remaining pellets after termination of incubations were submitted to sonication in methanol–chloroform, 1:1, by vol, followed by methanol and water. Less than 0.1% of the added radioactivity was found in the pellets, mainly the steroid substrate, when analyzed by TLC.

In control experiments incubations were conducted without steroid added and they were terminated by addition of 10 ml 150 mM NaCl followed by extraction with 60 ml of chloroform–methanol, 2:1 (by vol, for details see [15]). The aqueous layer was discarded, and the steroid was added to the organic phase which subsequently was evaporated to dryness at reduced pressure. The residue was dissolved in chloroform–methanol, 1:1 (by vol), transferred to a test tube and taken to dryness under a stream of nitrogen prior to analysis.

*Identification and quantitation of metabolites*

The identity of the metabolites formed was established by comparing chromatographic mobilities with those of authentic references in the TLC and GLC-systems used. Prior to GLC and/or GLC–RD the compounds were converted to trimethylsilylethers or methyloxime-trimethylsilylethers [16]. With the exception of 16α-hydroxy-4-androstene-3,17-dione the identities were also confirmed by GC–MS. Specific details on the identifications and chromatographic properties of the 6β-hydroxy- and 6-oxo-derivatives of 4-androstene-3,17-dione and 4-pregnene-3,20-dione respectively have been reported previously [11]. Yields were calculated by determination of $^{14}C$-activity after extraction of metabolites from the silica gel TLC plate.

## RESULTS

The influence of various extraction solvents on artefactual 6-oxygenation of 4-androstene-3,17-dione during work up of rat liver microsomal fractions are demonstrated in Table 1. Also included in Table 1 are the results obtained with boiled controls. As can be deduced from the table 4-androstene-3,6,17-trione was always obtained in higher yields than 6β-hydroxy-4-androstene-3,17-dione although the ratio between the derivatives varied somewhat. The yields of 6β-hydroperoxy-4-androstene-3,17-dione are not included in the table, since this derivative contributed on the average only to 5% of the total artefactual 6-oxygenation.

When 0.5, 2.0 and 8.0 mg of microsomal protein were extracted with 60 ml chloroform–methanol, 2:1 (v/v) and the organic phase was evaporated, a linear relationship between artefact formed and protein amount was obtained. Thus with 0.5 mg of microsomal protein the yield of 6β-hydroxy-androstene-3,17-dione was 2.30 nmol. With 2.0 mg it

Table 1. Artefactual 6-oxygenation of 4-androstene-3,17-dione (A) in male rat liver microsomal solvent extracts as related to extraction solvent

| | Derivatives formed* during work up of solvent extracts of | | | |
|---|---|---|---|---|
| | 105,000 *g* rat liver sediments | | Boiled controls | |
| Solvent | 6β-hydroxy-A | 6-oxo-A | 6β-hydroxy-A | 6-oxo-A |
| Chloroform–methanol 2:1 (by vol) | 1.85 | 4.45 | 2.30 | 4.50 |
| Chloroform–methanol 4:1 (by vol) | 2.15 | 3.10 | 2.80 | 5.00 |
| Dichloromethane | 0.90 | 2.55 | 0.80 | 3.10 |
| Ethylacetate | 1.55 | 4.20 | 1.00 | 3.50 |
| Diethyl ether | 0.90 | 1.65 | 1.55 | 3.70 |
| Methyl-isobutylketone | 0.40 | 0.60 | 0.55 | 0.55 |

*The values are expressed in nmol of product formed per mg of microsomal protein. One ml of a 10% (w/v) microsomal (105,000 *g*, sediment) suspension in a 0.1 M phosphate (pH = 7.4) buffer was diluted with 10 ml of a 150 mM NaCl solution and the mixture was extracted with 120 ml of the respective solvent. Unlabelled (1 μmol) and 0.15 MBq 4-$^{14}C$-labelled 4-androstene-3,17-dione dissolved in 100 μl of the respective solvent was added to the organic phase which was subsequently separated and evaporated at 25°C. Each residue was then analyzed by TLC (Silica gel 60; Merck). Solvent: chloroform–ethyl acetate, 2:1, by vol). Yields were calculated from $^{14}C$-recoveries after extraction of the steroids from the silica gel. Each figure represents the mean of 2 experiments.

was 9.60 nmol and with 8 mg it was 41.3 nmol; ($n = 3$). In these experiments 1 $\mu$mol of 4-$^{14}$C-labelled 4-androstene-3,17-dione was used.

To circumvent artefactual 6-oxygenation of 4-androstene-3,17-dione a combined organic extraction and direct liquid chromatography was attempted as presented in detail in Experimental. When applied to the boiled controls (0.5 ml 105 000 *g* sediment and 1 $\mu$mol of unlabelled 0.15 MBq 4-$^{14}$C-labelled 4-androstene-3,17-dione) the fraction eluted between 115 and 200% TCV contained only minute amounts of labelled 6$\beta$-hydroxy- and 6-oxo derivates of 4-androstene-3,17-dione according to subsequent TLC analysis. The yields corresponded to less than 0.25 nmol/mg of protein (less than 0.01% conversion) for 6$\beta$-hydroxy-4-androstene-3,17-dione and 4-androstene-3,6,17-trione in three different sets of experiments.

With the extraction/direct chromatography method the characteristics of the rat liver microsomal 6$\beta$-hydroxylase activity on 4-androstene-3,17-dione was reinvestigated (Figs 1–3). For comparison the artefactual formation of 6$\beta$-hydroxy-4-androstene-3,17-dione found during conventional work up (solvent extraction) has been included in the figures. To establish the concentration of NADPH in these experiments 0, 0.09, 0.28, 0.83, 2.5, 3.0 and 7.5 $\mu$mol of NADPH were added (dissolved in 0.5 ml of a phosphate buffer, 0.1 M, pH = 7.4) to 0.5 ml of the microsomal preparation. The yields of 6$\beta$-hydroxy-4-androstene-3,17-dione were 0.65, 1.35, 2.8, 4.85, 6.15, 5.80 and 6.10 nmol/mg of protein respectively when 1 mol of 4-androstene-3,17-dione was used as substrate. Similar yields were obtained with the NADPH generating system used. In control experiments no reduction was made in the amount of steroid added to the organic phase to compensate for the metabolism of androstene-3,17-dione in the parallel experiments with microsomes. In the latter experiments unmetabolized androstenedione was recovered in yields ranging between 80–90% depending upon the substrate amounts used.

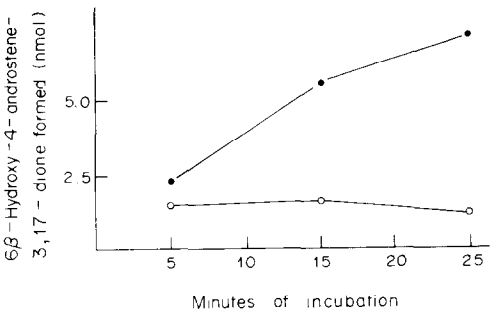


Fig. 2. Time dependency of the male rat liver microsomal 6$\beta$-hydroxylation of 4-androstene-3,17-dione. One $\mu$mol of unlabelled and 0.15 MBq 4-$^{14}$C-labelled steroid were added to the microsomal preparation (2 mg of protein) containing a NADPH-regenerating system. (●—●) Incubation experiments terminated with the extraction/direct liquid chromatography. (○—○) The labelled steroid was added to a chloroform–methanol 2:1, v/v, extract of incubated microsomes prior to evaporation. Results are expressed as nmol of product formed per mg of microsomal protein. For details see Fig. 1 and Experimental.

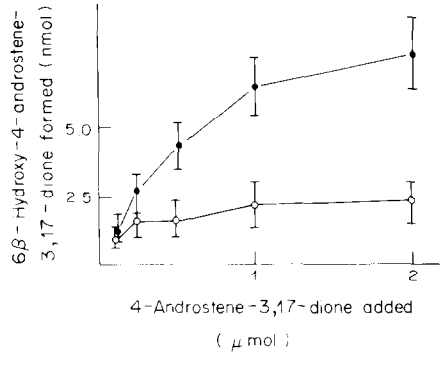


Fig. 1. Effects of various amounts of 4-androstene-3,17-dione upon 6$\beta$-hydroxylation in male rat liver microsomes incubated for 10 min at 37°C. Two mg of microsomal protein was used in a NADPH regenerating system. Values are expressed as nmol of product formed/mg of microsomal protein. Incubations were terminated with the extraction/direct liquid chromatography procedure (●—●) (Lipidex-1000, Packard Instrument Co.; solvent: methanol–water–*n*-butanol–chloroform, 60:40:7:3, by vol. For details see Experimental). (○—○) Experiments in which microsomes were first incubated for 10 min and then extracted with 60 ml of chloroform–methanol, 2:1 (v/v). 4-$^{14}$C-Labelled 4-androstene-3,17-dione was added to the organic phase prior to evaporation. Yields of 6$\beta$-hydroxy-4-androstene-3,17-dione were calculated after thin-layer chromatography separation (Silica gel 60 Merck, chloroform–ethyl acetate, 2:1; v/v). Means and ranges from five different experiments are presented.

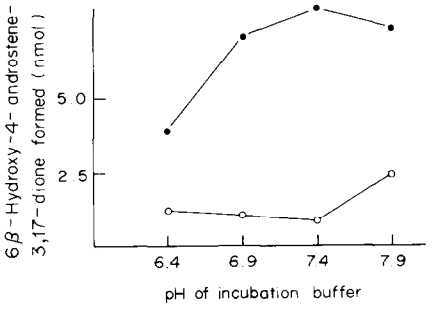


Fig. 3. Effects of incubation buffer pH on the 6$\beta$-hydroxylation of 4-androstene-3,17-dione in male rat liver microsomes incubated during 10 min at 37°C. A 0.1 M sodium phosphate buffer was used at the pH values shown in the figure. An NADPH-regenerating system was used in the same buffer but in these experiments NADPH was added from the start instead of NADP. One $\mu$mol of the unlabelled and 0.15 MBq of 4-$^{14}$C-labelled 4-androstene-3,17-dione were added to the microsomal preparation (2 mg of protein). (●—●) Incubation experiments terminated with the extraction/direct liquid chromatography procedure. (○—○) Experiments terminated by extraction with chloroform–methanol, 2:1 (by vol). For details see Fig. 1 and Experimental.

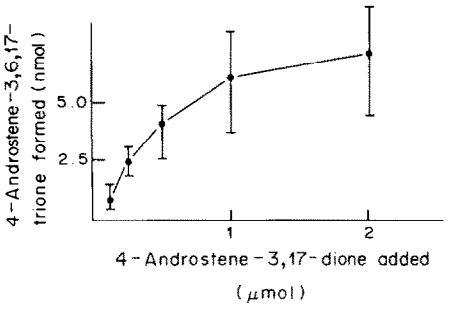


Fig. 4. Effects of amount of substrate added on the artefactual formation of 4-androstene-3,6,17-trione from 4-androstene-3,17-dione due to 3-imine formation during solvent evaporation. Two mg of microsomal protein was used with a NADPH regenerating system. Values are expressed as nmol of product formed/mg of microsomal protein. The labelled (and unlabelled) steroid was added to a chloroform–methanol, 2:1, (by vol) extract of incubated microsomal protein prior to evaporation. Yields were calculated as described in legends to Fig. 1. Means and ranges from five different experiments are presented.

As can be seen in Fig. 1 the artefactual 6β-hydroxylation may constitute as large a part of the total conversion as the enzymatic 6β-hydroxylation. Even when an apparent substrate saturation (1 μmol of 4-androstene-3,17-dione) was approached in the strict enzymatic conversion the artefactual 6β-hydroxylation amounted to 30% of the enzymatic one. The 6β-hydroxylase activity but not the artefact increased over time (Fig. 2). An increase in the pH of the incubation buffer increased the yields of artefactually formed 6β-hydroxy-4-androstene-3,17-dione whereas the activity of the 6β-hydroxylase proper decreased (Fig. 3).

The artefactual formation of 4-androstene-3,6,17-trione is illustrated in Fig. 4. Like the artefactual 6β-hydroxylation this reaction also mimicked a first-order enzymatic conversion as can be deduced from the figure. Whereas the amounts of 4-androstene-3,6,17-trione in the artefact producing experiments was on the average 2.5 times that of the amounts of 6β-hydroxy-4-androstene-3,17-dione (Table 1) no definite proof could be found for the formation of 4-androstene-3,6-17-trione in the extraction/direct liquid chromatography termination following incubations with NADPH fortified rat liver microsomal fractions. This trione comigrated with 3α- and 3β-hydroxy-5α-androstan-17-one in the TLC system used (Fig. 5). But subsequent GLC–RD allowed these compounds to be separated.

In experiments analogous to those with 4-androstene-3,17-dione, the microsomal formation of 6β-hydroxy-progesterone (direct extraction/liquid chromatography method) amounted to 0.17, 4.15, 4.85 and 5.05 nmol/mg microsomal protein when 0.05, 0.25, 0.5 and 1.0 μmol of labelled progesterone respectively were added. When the same amounts of progesterone were added to chloroform–methanol (2:1, v/v) extracts of microsomes followed by solvent evaporation (for details see Experimental) 1.25, 2.50, 3.25 and 3.50 nmol of 6β-hydroxy-progesterone were formed and 1.30, 2.75, 4.75 and 5.25 nmol of 6-oxo-progesterone respectively were isolated. Enzymatic and nonenzymatic 6-oxygenation of progesterone is shown in Fig. 6. In analogy with the results obtained with 4-androstene-3,17-dione no 6-oxo-derivative was obtained in the enzymatic conversion of progesterone (Fig. 6).

*Effects of carbon monoxide on microsomal 6β-hydroxylation of 4-androstene-3,17-dione and progesterone*

The inhibition by carbon monoxide of the rat liver microsomal 6β-hydroxylase acting on

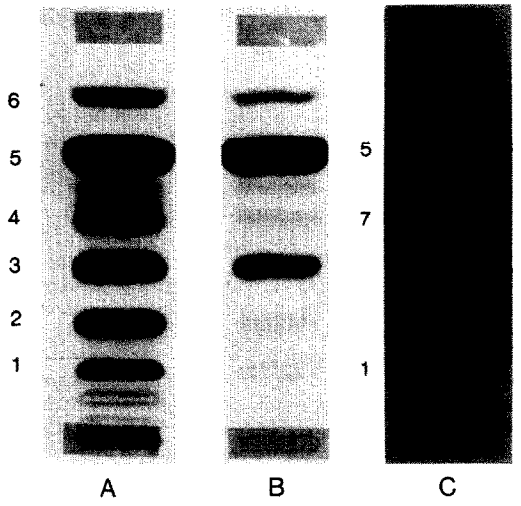


Fig. 5. Radioautography after TLC-analysis (Silica Gel 60 plates, 20 × 20 cm. Merck. Solvent system: chloroform–ethyl acetate, 2:1; v/v. The solvent system was allowed to rise 20 cm once) of: (A) An experiment where 1 μmol of unlabelled and 0.15 MBq 4-$^{14}$C-labelled 4-androstene-3,17-dione was added to 2 mg of male rat liver microsomal proteins and a NADPH regenerating system in 1 ml of a 0.1 M potassium phosphate buffer (pH 7.4). The incubation (10 min, 37°C) was terminated by the extraction/direct liquid chromatography procedure (see Fig. 1 and Experimental) and the fractions eluted between 115 and 200% of the total column volume were pooled and separated by TLC. The major metabolites subsequently identified (GLC–RD) were: 1. 6β-hydroxy-4-androstene-3,17-dione. 2. 16α-hydroxy-4-androstene-3,17-dione. 3. 17β-hydroxy-4-androsten-3-one. 4. 3α- and 3β-hydroxy-5α-androstane-17-dione. 5. 4-androstene-3,17-dione. 6. 5α-androstane-3,17-dione. (B) A similar experiment as in (A) but carbon monoxide was bubbled through the incubation mixture 2 min prior to and during the incubation. (C) An experiment with 2 mg of male rat liver microsomal proteins and a NADPH regenerating system in a 0.1 M potassium phosphate buffer (pH 7.4). The incubation (10 min, 37°C) was terminated by extraction with chloroform–methanol 2:1, (by vol); the aqueous phase was discarded and 1 μmol of unlabelled and 0.15 MBq 4-$^{14}$C-labelled 4-androstene-3,17-dione were added to the organic phase prior to evaporation. TLC analysis (see A) of the residue is shown. The metabolites were subsequently identified (GLC–RD, GC–MS) as: 1. 6β-hydroxy-4-androstene-3,17-dione. 5. 4-androstene-3,17-dione. 7. 4-androstene-3,6,17-trione.

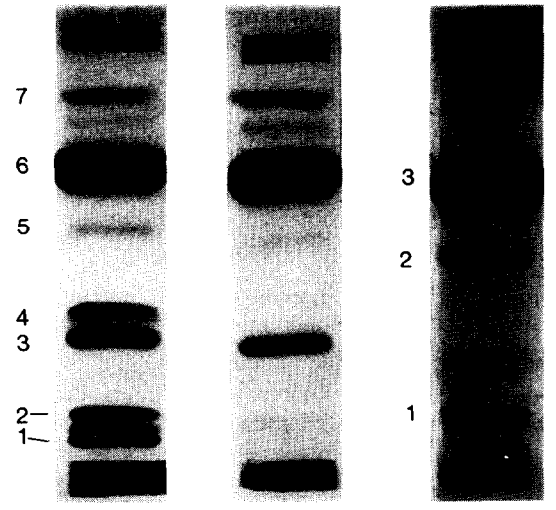


Fig. 6. Radioautography after TLC-analysis (Precoated Silica Gel 60 plates, 20 × 20 cm, Merck. Solvent system: chloroform–ethyl acetate, 6:1; v/v). (A) An experiment where 1 μmol of unlabelled and 0.15 MBq of 4-$^{14}$C-labelled 4-pregnene-3,20-dione were added to 2 mg of microsomal protein prepared from male rat liver and a NADPH generating system in 1 ml of 0.1 M potassium phosphate buffer (pH 7.4). The incubation (10 min, 37°C) was terminated by the extraction/direct liquid chromatography procedure (for details see Fig. 1 and Experimental). The fractions eluted between 140 and 300% of the total column volume were combined, evaporated and the residue was submitted to analysis by TLC. The major metabolites subsequently identified (GLC–RD and/or GC–MS) were 1. 16α-hydroxy-4-pregnene-3,20-dione. 2. 6β-hydroxy-4-pregnene-3,20-dione. 3. 20α- and 20β-hydroxy-4-pregnen-3-one. 4. 2α-hydroxy-4-pregene-3,20-dione. 5. 3β-hydroxy-5α-pregnan-20-one. 6. 4-pregnene-3,20-dione. 7. 5α-pregnene-3,20-dione. (B) A similar experiment as in (A) but carbon monoxide was bubbled through the incubation mixture 2 min prior to, and during the incubation. (C) An experiment with 2 mg of microsomal protein from male rat liver and a NADPH regenerating system in a potassium phosphate buffer (0.1 M, pH 7.4). The incubation (10 min, 37°C) was terminated by extraction with 20 times the volume of chloroform–methanol, 2:1, by vol. The aqueous phase was discarded and 1 μmol of unlabelled and 0.15 MBq 4-$^{14}$C-labelled 4-pregnene-3,20-dione were added to the organic phase prior to evaporation. The residue was submitted to TLC and the metabolites subsequently identified (GLC–RD, GC–MS) were as follows: 1. 6β-hydroxy-4-pregnene-3,20-dione. 2. 4-pregnene-3,6,20-trione. 3. 4-pregnene-3,20-dione.

4-androstene-3,17-dione is displayed in Fig. 5B. The actual inhibition when compared to control experiments like that in Fig. 5A was 95% (average of three experiments). This magnitude of 6β-hydroxylase inhibition was also obtained with progesterone as substrate (Fig. 6).

## DISCUSSION

The formation of 6β-hydroxy- and 6-oxo-derivatives together with 6β-hydroperoxy-derivatives of 3-oxo-4-ene steroids in boiled microsomes has been reported previously [9, 10]. We recently described the formation of these 6-oxygenated derivatives as a consequence of 3-imine formation between 3-oxo-4-ene-steroids and amines during solvent evaporation [11]. We also reported this to occur in organic solvent tissue extracts containing amines [12]. Since these reactions may obscure studies on the rat liver microsomal 6β-hydroxylase we first tried to avoid the artefactual 6-oxygenation by choosing a solvent which quenched 3-imine formation. As is clear from the result section this failed, although with methylisobutylketone less 6-oxygenated derivatives were obtained, possibly due to competition between the solvent and the ketosteroid for the amines. To circumvent 3-imine formation the direct liquid chromatography termination procedure was attempted. This procedure gave adequate recoveries of 4-$^{14}$C-labelled 4-androstene-3,17-dione and progesterone and their respective metabolites. When this method was applied to the boiled controls only minute amounts of 6-oxygenated derivatives of 4-androstene-3,17-dione or progesterone were found and no indication of imine-formation could be detected. This was interpreted as if a sufficient separation between the steroid substrate and the amines in the extract was achieved. Consequently direct extraction liquid chromatography was chosen as work up procedure after incubation with the microsomal preparations.

From the experiments with substrate concentration, time and pH it is clear that solvent extraction termination of incubations after evaporation produced an artefactual conversion of 4-androstene-3,17-dione to 6β-hydroxy-4-androstene-3,17-dione that resembled an enzymatic activity. Thus the extent of the artefactual reaction was proportional to the protein content. An apparent substrate saturation is obtained and the reaction yields varied with pH. The increased artefact formation at alkaline pH was probably due to an increased extractability of the amines in the organic solvent at elevated pH. The artefact however did not increase with incubation times as would be expected for an enzymatic activity. Although the artefactual formation of 6β-hydroxy-4-androstene-3,17-dione at substrate concentrations corresponding to enzyme saturation was clearly of a lesser magnitude than the enzymatic activity, it may present problems in quantitative work on 6β-hydroxylation. If artefactual 6β-hydroxylation is not excluded it may be that the differences obtained are due to changes in amine metabolism (i.e. amine concentrations) rather than a change in the 6β-hydroxylase activity. Boiled controls for each experiment can of course be used but since this is a tedious procedure it seems justified to propose the use of methodology that eliminates the major source of artefactual 6β-hydroxylation. This may be of special importance in pharmacological or physiological experiments where a 20–30% difference is to be established. Thus a 65% reduction in 6β-hydroxylation

was reported after carbon monoxide treatment of rat liver microsomes [9] but our present results show that when the steroid 3-imine mediated artefactual 6β-hydroxylation of progesterone and 4-androstene-3,17-dione was eliminated the extent of carbon monoxide inhibition of the enzymatic microsomal 6β-hydroxylation was 95%. Thus the present results seem to support the view that cytochrome $P_{-450}$ catalyzed 6β-hydroxylation of 4-androstene-3,17-dione and progesterone is the predominant if not the only 6β-hydroxylase activity for these steroids in the rat liver microsomal preparations studied. Although carefully looked for 4-androstene-3,6,17-trione could not be found after incubation with 4-androstene-3,17-dione, NADPH and rat liver microsomes followed by subsequent work up with the direct extraction/liquid chromatography method. Since this derivative was always present when the experiments were terminated with solvent extraction and evaporation the occurrence of 4-androstene-3,6,17-trione seems to indicate artefactual steroid 3-imine-formation and subsequent rearrangement during work up. Similar conclusions can be drawn from the experiments with progesterone as demonstrated by TLC-analysis (c.f. 4-pregnen-3,6,20-trione in Fig. 6).

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