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6-OXYGENATION OF 3-OXO-4-ENE-STEROIDS IN HIGH YIELDS AFTER 3-IMINE-FORMATION

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Summary—3-Imine formation between primary amines and 3-oxo-4-ene-steroids, followed by hydrolysis of the imines (either spontaneously during work up or induced by acetic acid) has been shown to cause 6-oxygenation of the steroids tested (17β-hydroxy-4-androsten-3-one, 4-androstene-3,17-dione, 4-pregnene-3,20-dione and 4-cholesten-3-one). The main products are the 6β-hydroxy- and the 6-oxo-derivatives of the respective steroid. These derivatives were identified by chromatographic mobilities and by gas chromatography–mass spectrometry. The formation of 6β-hydroperoxy-derivatives is suggested and these derivatives were tentatively identified. The highest yields of 6-oxygenated products (30–50%) were found when cadaverine and spermine were reacted with the steroids. The addition of reduced glutathione during hydrolysis of the steroid 3-imines of cadaverine, hexylamine and ethanolamine as well as addition of ascorbic acid during the hydrolysis of the steroid 3-imines of cadaverine substantially reduced the 6-oxygenation. Steroid 3-imine formation and hydrolysis which yields 6-oxygenated derivatives has also been shown to occur during work up (evaporation) of organic solvent extracts of rat liver microsomes (105,000 *g* sediments) to which 17β-hydroxy-4-androsten-3-one, 4-androstene-3,17-dione, 4-pregnene-3,20-dione or 4-cholesten-3-one respectively had been added. It is concluded that there is a risk that these organic reactions are mistaken for enzymatic conversions during *in vitro* investigations of 3-oxo-4-ene-steroids.

INTRODUCTION

We have previously shown [1] that 3-oxo-4-ene-steroids and many primary amines very easily react to form imine derivatives. During the spontaneous hydrolysis, characteristic of these labile compounds, a rearrangement of the steroid takes place. Two major metabolites were seen irrespective of the length of the steroid side-chain and regardless of the amine used [1]. Preliminary studies indicated that these compounds were also formed when 3-oxo-4-ene-steroids were added to organic extracts of rat liver subcellular fractions and the solvent was evaporated. The present study was undertaken to identify these compounds and to study the mechanisms of the rearrangement reaction(s) of steroid 3-imines.

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. Hexylamine, ethanolamine, putrescine, spermine and cadaverine were purchased from Sigma Chemical Company, St Louis, MO, U.S.A.

Radioactive compounds

[4-^{14}C]5-cholesten-3β-ol (55–61 mCi/mmol), [4-^{14}C]-17β-hydroxy-4-androsten-3-one (51–55 mCi/mmol), [4-^{14}C]4-pregnene-3,20 dione (52–55 mCi/mmol) and [4-^{14}C]4-androstene-3,17-dione (55–56 mCi/mmol), were purchased from the Radiochemical Centre, Amersham, England. [4-^{14}C]4-Cholesten-3-one was prepared from [4-^{14}C]5-cholesten-3β-ol as described previously [2]. All labelled steroids were freshly purified on the day preceding the experiments.

Unlabelled 4-androstene-3,17-dione, 4-androstene-3,6,17-trione, 6β-hydroxy-androstene-3,17-dione, 17β-hydroxy-4-androsten-3-one, 6β,17β-dihydroxy-4-androsten-3-one, 4-cholesten-3-one, 4-cholestene-3,6-dione, 6β-peroxy-4-cholesten-3-one, 4-pregnene-3,20-dione, 4-pregnene-3,6,20-trione and 6β-hydroxy-4-pregnene-3,20-dione were purchased from Steraloids Inc., NH, U.S.A.

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 [1] (and below). Unless otherwise stated ^{14}C-labelled steroids were diluted with unlabelled steroid to a specific activity of 5 mCi/mmol and used in the

List of abbreviations: Systematic names of the steroids referred to in the text by trivial names or abbreviations are as follows: testosterone, 17β-hydroxy-4-androsten-3-one; androstendione, 4-androstene-3,17-dione, progesterone, 4-pregnene-3,20-dione; 6-oxo-testosterone, 17β-hydroxy-4-androstene-3,6-dione, 6-oxo-androstendione, 4-androstene-3,6,17-trione; 6-oxo-progesterone, 4-pregnene-3,6,20-trione.

experiments. Yields of 6-oxygenated products were calculated based upon ^{14}C-recoveries from the starting amount of steroid added.

Synthesis of imine derivatives of steroids

Authentic 3-hexyl-imines of steroids were synthesized as described previously [1]. For most quantitative studies 10 mg of a free primary amine was dissolved in 60 ml of chloroform–methanol 2:1 (v/v) containing 100 μg of the respective steroid. The solution was taken to dryness (at reduced pressure) at 25°C. The residue was used for immediate analyses by TLC and/or GLC.

Acid hydrolysis of steroid-3-imines

The imine was dissolved in 3 ml of H_2O-acetic acid 1:1, by volume, refluxed for 30 min, allowed to cool and extracted with 15 ml of ethyl acetate. The organic layer was washed three times with 3 ml of distilled water and evaporated prior to analyses.

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, L-ascorbic acid (Vitamin C) and reduced glutathione, were purchased from Sigma.

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 presented in Table 1. 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

A Pye gas chromatograph (Model 104) equipped with a hydrogen flame ionization detector 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 or 260°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 were used. Six ml of a scintillation cocktail (Insta Gel, Packard) was added to an aliquot (50–250 μl) of the sample. ^{14}C-Labeled spots on TLC were located by exposing a sheet of Agfa Gaevert Osray M3 film to the plate for 1–14 days. GLC-radioactivity detection (GLC–RD) was accomplished with a Packard instrument 893.

Spectrometry

A Beckman Model 08-GD double beam grating spectrometer was used.

Derivatives prepared

Trimethylsilylethers were prepared as described by Makita and Wells[3]. Methyloxime–trimethylsilylethers and hydroxylamine–trimethylsilylethers were prepared as described by Axelsson and Sjövall[4].

Animals

Male Sprague–Dawley rats (200–220 g, Anticimex, Stockholm, Sweden) were kept for 2–3 days preceding the experiments. They were fed a standard pellet food (Anticimex) 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.

Rat liver subcellular fractions

The livers were excised and rinsed in a 0.25 M sucrose solution, pH 7.4. To 10 g of wet liver tissue was added 40 ml of the 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 were prepared in 0.1 M phosphate buffer, pH 7.4 with a NADPH regenerating system as described by Mode *et al.*[5]. The incubation were terminated and worked up as described previously [1].

RESULTS

Identifications

With the four different 4-^{14}C-labelled 3-oxo-4-ene-steroids studied, a similar transformation product pattern emerged upon TLC analysis after conden-

sation with various primary amines (for synthesis, see the Experimental section). Thus, apart from the parent steroid two major compounds which migrated in the TLC-systems used (see Table 1) were formed. With the exception of experiments with testosterone a minor component which migrated between the two major ones was observed. In each experiment where acid hydrolysis of the steroid imines was omitted, a substantial amount (10–30%) of ^{14}C-activity remained at the starting position of the silica gel plate, as previously reported for the 3-hexylimine derivative of testosterone [1]. Extraction of the component at the starting position followed by repeated TLC analysis revealed the presence of the parent 3-oxo-4-ene-steroid and the same migrating compounds as in the initial TLC analysis.

With 4-cholesten-3-one imine condensation followed by TLC analysis (system 6, Table 1) revealed spots at $R_f = 0.10$, 0.25 and 0.52. The same mobilities were found for authentic 6β-hydroxy-, 6β-hydroperoxy-4-cholesten-3-one and 4-cholestene-3,6-dione, respectively. These tentative identifications were next confirmed by GLC–RD (Table 1) and GC–MS.

The mass spectrum of the trimethylsilylether of the tentatively identified 6β-hydroxy-4-cholesten-3-one was identical to that published previously [2]. The 6-oxo-derivative fragmented like the authentic compound (Fig. 1).

After extraction from the silica gel the 6β-hydroperoxy-derivative decomposed to yield mainly the 6β-hydroxy- and 6-oxo-derivatives according to TLC and GLC.

The three principal conversion products of 4-cholesten-3-one (e.g. the 6β-hydroxy-, 6β-hydroperoxy- and 6-oxo-derivatives) were also obtained with progesterone and 4-androsten-3,17-dione. The pertinent chromatography data are shown in Table 1. The mass spectra of the TMS ethers of the respective 6β-hydroxy steroid were the same as previously published [6, 7, 8]. The spectra of the 6-oxo-steroids are displayed in Fig. 1. The fragmentation pattern for the tentatively identified 6-oxo-testosterone was dissimilar to those of the other 6-oxo-derivatives, possibly due to the 17β-trimethylsilylether group but the characteristic peaks at $m/z = 137$ and at $m/z = 243$ are present in the spectrum (Fig. 1). The methyloxime–trimethylsilyl (MO–TMS) derivatives of the tentatively identified 4-pregnene-3,6-20-trione, 4-cholestene-3,6-dione, and 4-androstene-3,6,17-trione, afforded GLC (Table 1) and GC–MS data

Table 1. Chromatographic properties of some 6-oxygenated 3-oxo-4-ene-steroids, synthesized by 3-imine formation

	TLC[a] R_f							GLC[b] t_r			
	1	2	3	4	5	6	7	1	2	3	4
17β-Hydroxy-4-androstene-3,6-dione	0.26			0.23					0.94	1.00 1.05 1.20 1.30	(1.35) 1.55
6β,17β-Dihydroxy-4-androsten-3-one	0.12	0.07	0.26	0.11					0.80		
17β-Hydroxy-6β-hydroperoxy-4-androsten-3-one	0.26			0.23							
4-Androstene-3,6,17-trione	0.55	0.37	0.53	0.32				0.86		0.85 0.90 1.05 1.10	(1.55) 1.75
6β-Hydroxy-4-androstene-3,17-dione	0.21	0.14	0.30	0.10					0.66		
6β-Hydroperoxy-4-androstene-3,17-dione	0.37			0.22							
4-Pregnene-3,6,20-trione		0.52	0.34	0.35	0.47			1.41		1.45 1.55 1.80 1.90	(2.65) 3.05
6β-Hydroxy-4-pregnene-3,20-dione		0.14		0.10	0.16				1.49		
6β-Hydroxyperoxy-4-pregnene-3,20-dione				0.25	0.38						
4-Cholestene-3,6-dione						0.52	0.70	3.17		3.35 3.55 4.15 4.40	(4.00) 4.60
6β-Hydroxy-4-cholesten-3-one						0.10			2.72		
6β-Hydroperoxy-4-cholesten-3-one						0.25	0.53				

Thin layer chromatography (TLC). (a) Silica Gel 60 plates (20 × 20 cm, Merck) were used in the solvents (1). chloroform–ethyl acetate, 2:1 (v/v); (2) chloroform–ethyl acetate, 4:1 (v/v); (3) benzene–ethanol, 9:1 (v/v); (4) toluene–ethyl acetate, 4:1 (v/v, developed 20 cm, twice); (5) chloroform–ethyl acetate, 6:1 (v/v); (6) benzene–ethyl acetate, 7:1 (v/v); (7) diethyl ether–cyclohexane, 12:1 (v/v). The solvent was allowed to rise once, unless otherwise stated. Separated compounds were detected by iodine vapour and/or by radioautography.

(b) A Pye gas chromatograph (Model 104) equipped with a flame ionization detector (FID) was used. The column (3 m × 4 mm) contained Supelcoport, 80–100 mesh, (Supelco) coated with 3% SP-2100 (Supelco). Carrier gas was nitrogen at a flow rate of 480 ml/cm^2/min. The derivatives were analyzed unprotected = 1, after conversion to trimethylsilylethers = 2, after conversion to methyloxime-trimethylsilylethers = 3 (this yielded four peaks all of which with identical mass spectra when analyzed by GC–MS), after conversion to hydroxylamine-trimethylsilylethers = 4 (this yielded one major and one minor peak with identical mass spectra. The figure within parentheses refers to the minor peak).

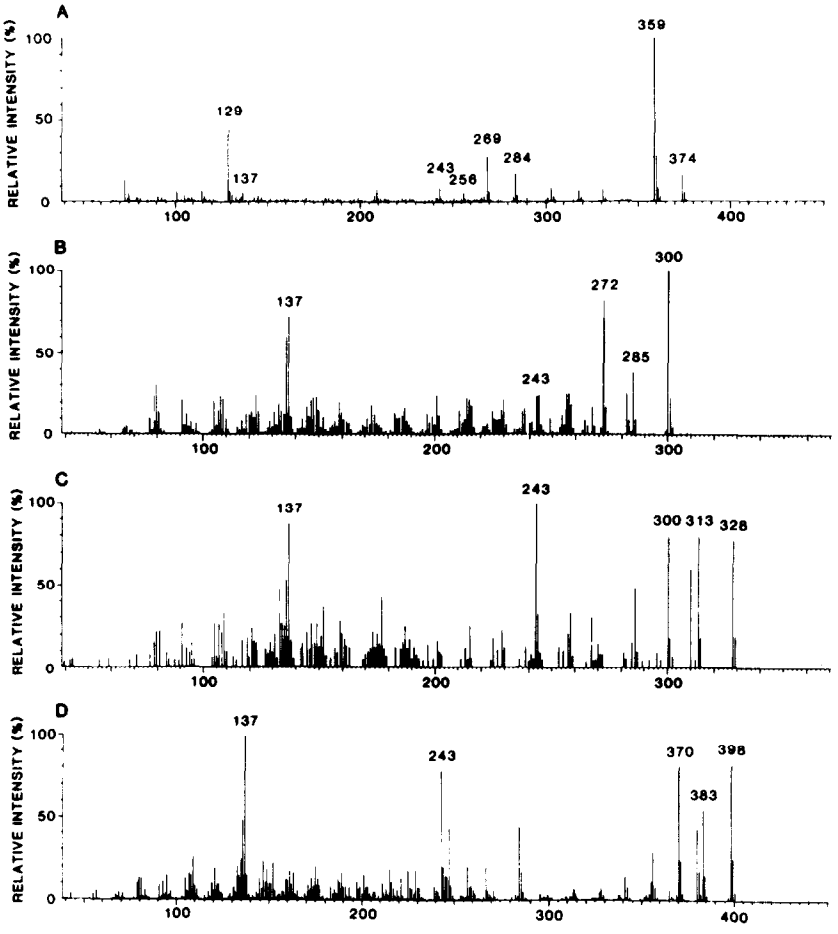

Fig. 1. Mass spectra obtained after gas chromatography mass spectrometry of (A) the trimethylsilylether of 6-oxo-testosterone (B) 6-oxo-androstenedione (C) 6-oxo-progesterone (D) 6-oxo-cholestenone. The steroids were synthesized via 3-imine formation and analyzed with a Finnegan Model 1020 instrument. For conditions, see the Experimental section.

(Table 2) which were identical to the corresponding derivatives of the reference compounds. The similarities between the fragmentation patterns of these and that of the MO–TMS derivative of 6-oxo-testosterone (Table 2) supported the tentative identification of the latter compound.

The identification of the 6β-hydroperoxy derivatives of 4-androstene-3,17-dione, progesterone and testosterone was based on analogies with the properties of authentic 6β-hydroperoxy-4-cholesten-3-one. This latter compound after extraction from the silica gel upon repeated TLC analysis and/or GC–MS

Table 2. Fragmentation pattern of methyloxime–trimethylsilyl (MO–TMS) derivatives of some 3,6-dioxo-4-ene-steroids, synthesized by 3-imine formation and analyzed by gas-chromatography–mass spectropmetry

	Parent steroid[a] *m/z* (% relative intensity)			
Ion[b]	17β-Hydroxy-4-androstene-3,6-dione	4-Androstene-3,6,17-trione	4-Pregnene-3,6,20-trione	4-Cholestene-3,6-dione
M	432 (100)	387 (100)	415 (100)	456 (100)
M-15	417 (20)	372 (15)	400 (15)	441 (20)
M-31	401 (60)	356 (45)	384 (45)	425 (80)
M-47	385 (20)	340 (20)	368 (20)	409 (45)
M-63	369 (20)	324 (15)	352 (15)	393 (30)
	168 (65)	168 (45)	168 (40)	168 (90)
	129 (20)			

[a]The steroids were converted to methyloxime-trimethylsilylethers prior to analysis.
[b]Major fragments are listed. A Finnigan instrument was used. For conditions see Experimental section.

analysis gave rise to the 6β-hydroxy and the 6-oxo-derivatives. The corresponding results were obtained with the tentatively identified 6β-hydroperoxy-derivatives of 4-androstene-3,17-dione and progesterone (for chromatographic properties see Table 1). A suspect 6β-hydroperoxide of testosterone was found to migrate like 6-oxo-testosterone in the TLC-systems used, since extraction of the 6-oxo-testosterone region followed by trimethylsilylation and GC–MS led to the identification of 6β-hydroxy-testosterone as well as 6-oxo-testosterone.

Factors affecting formation of 6-oxygenated 3-oxo-4-ene-steroids

Amine structure. Several primary amines were tried, and the highest yields of 6-oxygenated steroids were found after reaction with aliphatic di- and polyamines (i.e. those amines reported [1] to be most reactive towards oxo-steroids) and subsequent work up by LC or TLC. Spermine and cadaverine were found to be the most potent amines in this respect and yielded 30–50% of 6-oxygenated steroids when a 100-fold molar excess of amine was evaporated at room temperature together with the steroid in chloroform–methanol, 2:1 (v/v). In experiments with ethanolamine and progesterone that were standardized to yield approx 12% of 6-oxygenated derivatives five consecutive experiments gave the following mean proportions: 6-oxo-progesterone–6β-hydroxy-progesterone–6β-hydroperoxy-progesterone, 6:4:1. When diaminopyridine or triethylamine replaced cadaverine or spermine, only small amounts of 6-oxygenated steroids could be seen (less than 1%). Similar low yields were obtained when 10 μl of 0.1 M NaOH and 10 mg of diaminopyridine were added to 100 μg of testosterone in 60 ml chloroform–methanol, 2:1 (v/v) and the mixture taken to dryness at 25°C.

Temperature. Mixtures of 100 μg testosterone and 10 μl cadaverine in 60 ml chloroform–methanol, 2:1 (v/v) were taken to dryness at 0, 30, 50 and 100°C respectively. The residues were analyzed by TLC. This gave rise to 6-oxygenated derivatives in yields of 23, 26, 27 and 30%, respectively. In similar experiments where the residues were submitted to acid hydrolysis (acetic acid–H_2O, 1:1, 110°C for 30 min) and extracted with diethyl ether prior to analysis by TLC, the yields were 13, 18, 28 and 32%, respectively. In control experiments without amine, less than 1% of 6-oxygenated testosterone derivatives were found.

Addition of antioxidants. Cadaverine (10 μl) and 100 μg steroid were dissolved in 60 ml methanol–chloroform and taken to dryness at 100°C. The residue was dissolved in 5 ml aqueous acetic acid (50%) containing 500 mg ascorbic acid and refluxed in air for 30 min. The mixture was allowed to cool and then extracted twice with 30 ml diethyl ether. The aqueous layer was discarded and the organic phase was washed with water until neutral and then taken to dryness. This yielded 1.0–3.6% 6-oxygenated derivatives with testosterone as substrate and 8–10% with 4-cholesten-3-one. Controls without ascorbic acid yielded more than 30% 6-oxygenated derivatives with both steroids. When 500 mg of reduced glutathione was used instead of ascorbic acid the yields of 6-oxygenated steroids were less than 2% with the two test steroids. In two similar experiments where ethanolamine replaced cadaverine, addition of reduced glutathione (500 mg) to the aqueous acetic acid yielded 1.0 and 1.3% of 6-oxygenated testosterone, respectively. Experiments without glutathione yielded 20.3 and 22.9% of 6-oxygenated derivatives respectively. When authentic testosterone-3-hexylimine was refluxed in the aqueous acetic acid the extent of 6-oxygenation was 42% but when reduced glutathione was included yields dropped to less than 2%.

Testosterone-3-hexylimine hydrolysis. When authentic (GC–MS-verified) testosterone-3-hexylimine was dissolved in chloroform a maximum u.v.-absorption was noted at 245 nm (ϵ = 16,500). When 20 μl of concentrated HCL was added to this solution a shift in the absorption was noted (max = 265 nm, ϵ = 20500).

Reaction of the 6-oxygenated-derivatives of 3-oxo-4-ene steroids with primary amines. [4-^{14}C]6β-Hydroxy-4-cholesten-3-one or [4-^{14}C]6β-hydroxy-progesterone was reacted with 10 μl of spermine or 10 μl of hexylamine in 60 ml chloroform–methanol, 2:1, v/v, evaporated to dryness and subsequently analyzed by LC and/or TLC. No polar or non-polar derivatives were seen except 6-oxo- (60%) or 6β-hydroxylated (40%) derivatives of the respective steroid. When the 6-oxo-derivatives of the respective steroid were reacted in the same way, more than 99% was found in a highly polar fraction (spermine) or a non-polar fraction (hexylamine) as based upon the chromatographic mobilities on LH-20 gel in the solvent system. 2.2.4-Trimethylpentane–benzene–methanol, 90:5:5 (by vol, progesterone), or on Lipidex-1000 in the system methanol–water–*n*-butanol–chloroform, 70:30:7:3 (by vol, 4-cholesten-3-one).

Experiments in vitro with rat liver microsomal preparations. 4-^{14}C-Labelled and 100–500 μg unlabelled 4-androstene-3,17-dione, 4-pregnene-3,20-dione, 17β-hydroxy-4-androsten-3-one and 4-cholesten-3-one, respectively were each added to a chloroform–methanol, 2:1 (by vol) extract of the standard rat liver microsomal preparation (0.5 ml, 4–6 mg of proteins) where the aqueous layer had been discarded. The organic layer was subsequently taken to dryness and the residue was analyzed by LC, TLC and GLC–RD. Control experiments consisted of organic extracts of the incubation buffer. This revealed that the biological extracts contained compounds that reacted with the 3-oxo-4-ene-steroids yielding derivatives with chromatographic properties (TLC, GLC–RD) identical to the 6-oxygenated steroids shown in Table 1. These compounds could not

be found in the buffer controls (less than 0.05%). The artefactual transformation of 3-oxo-4-ene-steroids to 6-oxygenated derivatives was not completely abolished with the steroid amounts tested. For instance when 1 μmol of 4-androstene-3,17-dione was added to the organic extract of the rat liver microsomal preparation, the yields of 6-oxygenated derivatives ranged between 1.2–2.0% ($n = 5$).

DISCUSSION

Our results show that steroid-3-imine formation enhances 6-oxygenation of 3-oxo-4-ene-steroids. The identification made of the compounds formed rests primarily on GC–MS data, as well as TLC and GLC results which agree with reference compounds and previously published results [2, 6, 7, 8].

It appears that a 6β-hydroperoxide-steroid is formed along with the 6β-hydroxy- and 6-oxo-derivatives and the former compound might be the precursor of the other two. With the reference available (i.e. 6β-hydroperoxy-4-cholesten-3-one) we could establish the identity of this compound which was formed from imine derivatives. The imine derivatives of the other steroids gave rise to compounds analogous to 6β-hydroperoxy-4-cholesten-3-one in that they decomposed to mainly the 6β-hydroxy- and 6-oxo-derivatives. This is in accordance with the findings of Yu and Tan[9] on the 6β-hydroperoxy-derivatives of androstendione and progesterone.

From the present study it is clear that artefactual formation of 6-oxygenated derivatives of 3-oxo-4-ene-steroids may occur in evaporated organic solvent extracts of biological samples due to imine formation. Subsequent 6-oxygenation is of a magnitude that may easily be mistaken for enzymatic activity. Tan *et al.*[9,10] have reported high degrees of 6-oxygenation of 4-androstene-3,17-dione and 4-progesterone-3,20-dione even in boiled controls of bovine adrenal mitochondria. Since these authors terminated their experiments with solvent extraction and evaporation it is conceivable that these results were due to imine formation and subsequent rearrangement. The data presented in the present investigation may cast some doubt on the specificity of previous studies on the 6β-hydroxylation of 3-oxo-4-ene steroids *in vitro* when the results rest primarily on the quantitation of these compounds after solvent extraction and evaporation.

The introduction of oxygen at C-6 in 3-oxo-4-ene-steroids has been suggested to be facilitated via an enolization leading to a 3,5-diene structure [11]. Reaction of 3-oxo-4-ene-steroids and secondary amines has been shown to yield a 3,5-diene structure [12]. It thus seems conceivable that our steroid-imine derivatives during the protonation of the imine structure—believed to be the rate limiting step in imine hydrolysis [13]—undergo a rearrangement to form a 3,5-diene intermediate suitable for attack by oxygen. This interpretation was also supported by our u.v. absorbtion data on the testosterone–hexylimine [14]. Our results also show that base catalysis *per se* (known to facilitate enolization of 3-oxo-4-ene-steroids [15]) does not give rise to 6-oxygenated products to the same extent as with organic primary amines.

That the 6-oxygenation of conjugated steroid-3-imines takes place via a direct oxidation by oxygen is indicated by the results with two "antioxidants", e.g. ascorbic acid and reduced glutathione, which both quenched the reaction. However our experiments do not exclude the possibility that ascorbic acid (or dihydroascorbic acid) and reduced glutathione acted through a reaction with the steroid-3-imine thus blocking 6-oxygenation.

The proposed reaction scheme for the introduction of oxygen at C-6 is in close agreement with that proposed for the enzymatic 6β-hydroxylation of 3-oxo-4-ene-steroids [15] where molecular oxygen is introduced via a 3,5-dienol structure. In a study on the microbial 6β-hydroxylation of 4-androstene-3,17-dione, Holland and Diahow [11] showed a rate determining step prior to enolization and proposed that this step might be the initial substrate-enzyme binding. Interestingly oxo-steroids have been shown to form Schiff's bases with lysine residues in proteins in aqueous solutions [16, 17]. Our results demonstrate that 6β-hydroxy-testosterone does not easily form 3-imine derivatives. It is therefore tempting to speculate that an enzyme-bound 6β-hydroxy-4-ene-steroid-3-imine is easily hydrolyzed, thus favouring the overall reaction.

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