Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 825–834. Pergamon Press. Printed in Great Britain

SYNTHESIS AND PROPERTIES OF TWO NEW STEROID HYDROPEROXIDES: AN ALTERNATIVE PATHWAY FOR THE FORMATION OF 6β-HYDROXYPROGESTERONE

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(*Received* 12 *November* 1976)

SUMMARY

Two new steroid hydroperoxides, 6β-hydroperoxyprogesterone and 6β-hydroperoxyandrostenedione, were synthesized from the corresponding 5-ene-3-ketosteroids chemically by oxygenation in an aprotic solvent in the presence of dibenzoyl peroxide as a catalyst, and biochemically by using horseradish peroxidase or bovine adrenal mitochondria as enzyme sources. The absolute stereochemical configuration of the substituent at C-6 was established by NMR and by reduction to the known 6β-hydroxy compounds. These hydroperoxides undergo thermal decomposition giving rise to a spate of other products, as shown by gas chromatography (GC), mass spectrometry (MS), and combined GC–MS. They also exhibit a characteristic absorption maximum at 398 nm when treated with concentrated sulfuric acid. 6β-Hydroperoxyprogesterone binds to adrenal cytochrome P-450 and it is also readily converted into 6β, 11α-dihydroxyprogesterone by cultures of *Rhizopus arrhizus*. A new pathway for the biosynthesis of 6β-hydroxyprogesterone is proposed.

INTRODUCTION

In the living cell, molecular oxygen assumes two important functions: first it acts as the ultimate hydrogen or electron acceptor by stepwise reduction to H_2O or H_2O_2; secondly, molecular oxygen acts as a chemical reactant by transforming dietary nutrients into cellular constituents and other biologically important substances. It can do this either by incorporation of one oxygen atom, or both of its oxygen atoms. The enzymes catalyzing these oxygen incorporations are called respectively mono-oxygenases (or mixed-function oxidases) and di-oxygenases [1, 2]. It is generally taken for granted that the introduction of hydroxyl groups into the steroid molecule is catalyzed by the former type of enzymes. Ringold[3] has proposed in 1962 an ionic mechanism for hydroxylations taking place at activated carbon atoms, e.g. at position 6 in 4-ene-3-keto steroids. Base-catalyzed enolization of the conjugated ketone, followed by electrophylic attack of an OH^+ cation at position 6 would preponderantly lead to a 6β-hydroxy-steroid. However, is the above mechanism the only valid one to explain 6β-hydroxylation in steroids? Can we totally exclude the participation of enzymes, other than monooxygenases? In the oxidation of unsaturated fatty acids such as in the prostaglandin precursor, arachidonic acid, dioxygenases constitute the active enzymes [4]. One can certainly say that the possible participation of dioxygenase or peroxidase type enzymes in the introduction of oxygen functions into the steroid molecule has been a neglected area of research. A case in point is the aforementioned introduction of a 6β-OH group. Steroid-6β-hydroxylase activity has been described for rat hepatic microsomes [5], adrenal and liver whole homogenate of guinea pig [6], human [7] and sheep foetal blood cells [8], and human placental tissues [9].

We thought it worthwhile to look into the possible formation of 6β-dioxygenated compounds in steroid biosynthesis. Fieser[10] has already shown in 1955 that β, γ-unconjugated ketones easily undergo autoxidation to yield γ-hydroperoxy-α, β-unsaturated ketones. In this way, 10β-hydroperoxyestrenes were synthesized by Layne[11]; 14β-hydroperoxyandrostenes by Campbell[12] and Afonso[13], while 11β-hydroperoxides were synthesized by a French group [14]. On the other hand, the only known 6-hydroperoxysteroids appear to be 6β-OOH-cholestenone, synthesized by Fieser[10], and its 6α-epimer, isolated by Cox in 1965 [15].

In this paper we report the synthesis of two new 6β-OOH-steroids, namely 6β-OOH-progesterone and 6β-OOH-androstenedione, and describe some of their properties. We further present evidence that 6β-hydroxyprogesterone, a steroid which has been found in adrenal tissue, can also be formed via a pathway

Abbreviations: 6β-OOH-progesterone = 6β-hydroperoxy-4-pregnene-3,20-dione; 6β-OH-progesterone = 6β-hydroxy-4-pregnene-3,20-dione; androstenedione = 4-androstene-3,17-dione; PHMB = *p*-hydroxymercuribenzoate; t.l.c. = thin layer chromatography.

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which does not necessarily require the presence of a 6β-hydroxylase.

EXPERIMENTAL

Materials

Pregnenolone and dehydroepiandrosterone were purchased from Organon (Holland). [4-^{14}C]-Pregnenolone was from New England Nuclear Co. Other reference steroids such as 6β-hydroxyprogesterone, 6-oxoprogesterone, 5-pregnene-3,20-dione, progesterone, 4,6-pregnadiene-3,20-dione, 4-androstenedione, 5-androstene-3,17-dione, 6β-hydroxyandrostenedione, 6-oxo-androstenedione, horse radish peroxydase (type II) and co-enzymes were from Sigma Chemical Co. Fresh bovine adrenals were obtained from a local slaughter house. Organic solvents were freshly distilled prior to use. Silicagel HF-254 for t.l.c. was from Merk AG, Darmstadt.

Methods

Gas chromatography. Steroid samples were analyzed on a Hewlett–Packard 7610A instrument as described previously [16].

Gas chromatography–Mass spectrometry. Combined gas chromatography–mass spectrometry was performed with a AEI MS-30 double beam instrument. The steroid solutions were injected into a coiled 150 × 0.3 cm glass column packed with a dual layer, consisting of 3% of OV-1 and 3% of OV 210 (1:1) on 100–200 mesh Gaschrom Q.

Infrared spectroscopy. Infrared spectra of the solid steroids were recorded on a Perkin–Elmer double beam Model 457 grating spectrometer equipped with a beam condenser. Steroid–KBr weight ratios were maintained at about 1:100. For the dilution studies, the hydroperoxysteroids were dissolved in chloroform.

NMR-spectrometry. The steroid samples were dissolved in deuterated chloroform with tetramethylsilane added as an internal standard and the NMR spectra were recorded on a Varian A-60 instrument at room temperature.

Mass Spectrometry. The steroids to be analyzed were dried over phosphorus pentoxide in a dessicator under vacuum for 20 h. Mass spectra were obtained by introduction of the sample via the direct inlet probe into the ion source of a Hitachi RMU-6E instrument.

Melting point measurement. Melting points were determined on a Kofler hot stage under microscopic magnification at barometric pressure and were not corrected.

Radioactivity measurements. Radioactivity was measured with a Packard-Tri-Carb Model 3380 or a Nuclear-Chicago Mark I liquid scintillation counter. Radioactive material, dissolved in 10–100 μl of dioxane was mixed with 15 ml of scintillation solution (3.0 g of PPO and 100 mg of POPOP in 1 liter of distilled toluene). Efficiency for ^{14}C in both instrument was in the order of 90%.

Thin layer chromatography. Thin layer chromatography (t.l.c.) of steroids was conducted on 5 × 20 cm or 20 × 20 cm glass plates, coated with 0.2 mm or 0.5 mm Silicagel HF_{254} (Merck, Darmstadt). The solvent system was toluene–ethyl acetate (4:1, v/v). The spots were examined first under ultraviolet light at 254 nm, and then after spraying with a 50% aqueous solution of sulfuric acid and heating at 110° for 2 min. The hydroperoxide spots were also visualized with a 0.3% solution of N, N-dimethyl-*p*-phenylenediamine dihydrochloride [17].

Preparation of 5-pregnene-3,20-dione. Pregnenolone was oxidized as described by Djerassi *et al.*[18]. To a cold (10°) solution of 10 g of pregnenolone in 1,500 ml of acetone (stabilized by distillation over permanganate) was added rapidly with stirring under nitrogen atmosphere 15 ml of Jones reagent [19]. After 5 min the reaction mixture was diluted with 2 l. of ice water. The white precipitate was recrystallized from acetone–water to give 7.47 g of shiny colorless crystals, m.p. 150–156°; I.R., 1690, 1220, 995, and 796 cm^{-1}. Gas chromatography of this product at 220° gave a single peak with the same retention time as progesterone.

Preparation of 5-androstene-3,17-dione. Dehydroepiandrosterone (10 g), dissolved in 1500 ml of stabilized acetone was oxidized in a similar manner with Jones' reagent. Recrystallization from acetone–water yielded 2.50 g of white crystals. m.p. 125–129°; I.R., 1738, 1708, 1052, 1012, and 800 cm^{-1}. Gas chromatography at 220° of this compound showed a single peak with the same retention time as 4-androstene-3,17-dione.

Preparation of 6β-hydroperoxy progesterone. 5-Pregnene-3,20 dione (5.5 g) was dissolved in a mixture of 150 ml of benzene and 60 ml of cyclohexane in a flask equipped with a reflux condenser and a sparger tube for bubbling oxygen through the liquid. The temperature of the solution was kept at 46° by a heated paraffin bath. Dibenzoyl peroxide (0.15 g) was added to the solution and the reaction mixture was oxygenated for 66 hours. The precipitate thus formed was collected by suction filtration. Recrystallization of this product from acetone–water gave 2.294 g of white waxy flakes of 6β-hydroperoxyprogesterone. M.p. 164–168°C; I.R., 3300, 1710, 1660, 1235, and 880 cm^{-1}; $[\alpha]_D^{22} = +12.09°$ (c = 1%, chloroform), $\lambda_{max}^{ethanol}$: 237 nm, $\lambda_{max}^{H_2SO_4}$: 398 nm, $\epsilon_{1cm}^{1\%} = 118$ at 237 nm (ethanol). CH analysis: calculated for $C_{21}H_{30}O_4$, C, 72.83; H, 8.67; found: C, 72.66, H, 8.93; mass spectrum, *m/e* 346 (M^+). Reduction of the 6β-hydroperoxy progesterone by a solution of 10% potassium iodide and acetic acid gave a product, which was identical in all aspects with authentic 6β-hydroxyprogesterone, m.p. 160–164°, I.R., 3420, 1675, 1412, 1352, 1112, and 708 cm^{-1}. Gas chromatography of the latter compound at 220° showed two peeaks with $t_R = 1.463$ and 1.732 (relative to progesterone). $\lambda_{max}^{H_2SO_4} = 351$ nm. Further concentration of the mother liquid of the oxygenation reaction gave a yellow siropous residue (3.1 g), consisting of a mixture of 6-keto-progesterone,

6β-hydroxyprogesterone, 6β-hydroperoxyprogesterone, and 4 other unidentified steroids. Attempted epimerization of the 6β-hydroperoxyprogesterone to its 6α-epimer by methanolic KOH [20] was not successful as our analysis of the reaction product by sulfuric acid spectra, t.l.c., optical rotation, I.R., m.p., gas chromatography, and chemical reduction showed that the recovered product was the unchanged 6β-hydroperoxy compound.

Preparation of 6β-hydroperoxyandrostenedione. The preparation of 6β-hydroperoxyandrostenedione was performed in the same manner as that of 6β-hydroperoxyprogesterone. Thus, 600 mg of 5-androstene-3,17-dione was oxygenated to yield 224 mg of 6β-hydroperoxyandrostenedione. M.p. 165–172°, I.R., 3320, 1735, 1655, 1414, 875, and 720 cm^{-1}, $[\alpha]_D^{22} = +11.47°$ (c = 1%, chloroform), $\lambda_{max}^{ethanol}$ = 237 nm, $\lambda_{max}^{H_2SO_4}$ = 398 nm, and $\epsilon_{1cm}^{1\%}$ = 148.0 at 237 nm (ethanol). CH analysis: calculated for $C_{19}H_{26}O_4$: C, 71.61, H, 8.16; found: C, 71.46, H, 8.28. Reduction of the 6β-hydroperoxyandrostenedione gave a product with m.p. 164–170°; I.R., 3480, 1728, 1650, and 875 cm^{-1}. $\lambda_{max}^{H_2SO_4}$ = 352 nm; identical in all aspects with authentic reference 6β-hydroxyandrostenedione.

Preparation of [4-^{14}C]-5-pregnene-3,20-dione. Recrystallized pregnenolone (800 mg) dissolved in 106 ml of acetone (distilled from permanganate), and 20 μCi of [4-^{14}C]-pregnenolone (0.12 mg) was oxidized by the Jones reagent as described before. The radioactive product had a m.p. of 154–158°; I.R., 1695, 1225, 957, and 798 cm^{-1}. It had a specific activity of 55,500 d.p.m./mg.

Absorption spectra in concentrated sulfuric acid. A few micrograms of the steroid were added to 4.0 ml of concentrated sulfuric acid. After mixing the solution with a glass rod for one minute, the sample was stored in the dark for 2 h at room temperature as described by Kornel and Motohashi[21]. The coloured solution was then scanned between 220 and 600 nm, using a Beckman model 24 spectrophotometer.

Spectrometric measurements of the horseradish peroxidase reactions. Time-course incubations of 5-pregnene-3,20-dione and 5-androstene-3,17-dione in 0.06 M Tris–HCl buffer, pH 7.4, in the presence of horseradish peroxidase were carried out using a Beckman Model 24 spectrophotometer. The sample and reference cells were maintained at 4°. In some samples, H_2O_2 was added in a concentration of 2×10^{-7}M.

Incubation of [4-C^{14}]-5-pregnene-3,20-dione with horseradish peroxidase. The incubation medium consisted of a 0.06 M Tris–HCl pH 7.4 buffer. The radioactive steroid (10 mg) was dissolved in dioxane together with 30 mg of carrier 5-pregnene-3,20-dione. Horseradish peroxidase was dissolved first in 2.6 ml of the buffer medium, at a concentration of 55.7 mg per ml. Other compounds used in the incubations were H_2O_2 (8×10^{-5}M), PHMB(4×10^{-3}M), and catalase (39 mg in 5.0 ml of the incubation medium). The incubations were conducted in an ice bath for 60 min with shaking either in air, or in a closed high vacuum system under argon atmosphere [16]. The reaction was terminated by extraction with 25 ml of a chilled ether–ethyl acetate (1:1, v/v) solution.

Incubation of [4-^{14}C]-5-pregnene-3,20-dione with bovine adrenal cortex mitochondria. Fat, medulla, and the adhering capsule were removed from 20 adrenal glands. The brownish tissue was suspended in 80 ml of 0.25 M sucrose. The suspension was filtered on a cheese cloth and the cortex pieces were resuspended in 200 ml of 0.154 M KCl. These washings were repeated thrice. The pieces were then blotted on filter paper, and resuspended in 200 ml of 0.25 M of sucrose and homogenized in a Virtis blender. The homogenate was filtered again over cheese cloth, resuspended in 300 ml of 0.25 M of sucrose, and the suspension was treated in a Potter–Elvehjem homogenizer for a total of 12 passes in a cold room with intermittant cooling of the glass tube in an ice bath to offset the heat generated by the friction. The homogenate was then centrifuged at 3020 *g* for 20 min at 0°. The supernatant (225 ml) was re-centrifuged at 22,000 *g* at 0° for 15 min. The mitochondrial pellet was resuspended in 45 ml of 0.25 M of sucrose, treated with a Potter–Elvehjem homogenizer and re-centrifuged at 22,000 *g* at 0°C for 30 min. Three-fourth of the pellet was resuspended and homogenized in 60 ml of medium (100 parts of 2% bovine serum albumin in 0.15 M NaCl of pH 7.4, 20 parts of 0.15 M of glycylglycine pH 7.4 buffer, and 1 part of 0.11 M $MgCl_2$), and a NADPH-generating system consisting of 47 mg of $NADP^+$, 67 mg of glucose-6-phosphate, and 10 units of glucose-6-phosphate dehydrogenase. To each of the incubation flasks containing steroids, 10 ml of this medium was added. The incubations were carried out at room temperature for 1 h on a shaker. They were terminated by the addition of 10 ml of chilled methylisobutylketone to the mixture with vigorous shaking.

Extraction and analysis of the incubation products. The organic and aqueous phases containing the incubation products were separated in a funnel, and the aqueous layer extracted twice more with an equal vol. of fresh organic solvent. The organic extracts were pooled, washed with sodium hydrogen carbonate, water, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. To each vial was added 0.4 ml dioxane to dissolve the product. Aliquots of 10 μl of each of the samples were mixed with 15 ml of scintillation solution and the radioactivity counted. A further aliquot of 50 μl was spotted on a 5 × 20 or 20 × 20 cm t.l.c. plate coated with 0.5 mm of silicagel. On each margin of the t.l.c. plate, reference steroids such as 6-ketoprogesterone, 6β-hydroxyprogesterone, 6β-hydroperoxyprogesterone, 5-pregnene-3,20-dione, and progesterone were also applied on a single spot. The t.l.c. plate was irrigated twice with toluene–ethyl acetate (4:1, v/v) as a solvent system. The plate

was then viewed under an U.V. lamp at 254 nm. The steroid bands corresponding to 6β-hydroxyprogesterone, 6β-hydroperoxyprogesterone, 6-ketoprogesterone, 5-pregnene-3,20-dione, and the origin were scraped off the plate and eluted with acetone: methanol (3:1, v/v). The eluates were filtered into scintillation vials, dried under a stream of nitrogen, mixed with 15 ml of the scintillation solution, and the radioactivity was counted.

Trapping of radioactive 6β-OOH-progesterone by incubations of a mixture of C^{14}-labelled progesterone and non-radioactive 6β-OOH-progesterone with either bovine adrenal cortex mitochondrial or microsomal fractions. The bovine adrenal cortex mitochondrial fraction was obtained in the manner described earlier. The mitochondrial pellets were homogenated in 0.06 M Tris–HCl pH 7.2 buffer to a final vol. of 29.0 ml. The microsomal fraction was obtained by further centrifuging the 22,000 *g* supernatant at 40,000 rev./min (105,000 *g*) for 60 min at 0°. The pellets were homogenized in 0.154 M KCl in a Potter–Elvehjem homogenizer, and re-centrifuged at 105,000 *g* for another h. The microsomal pellets were dissolved in 0.06 M Tris–HCl pH 7.2 buffer to a final vol. of 24 ml.

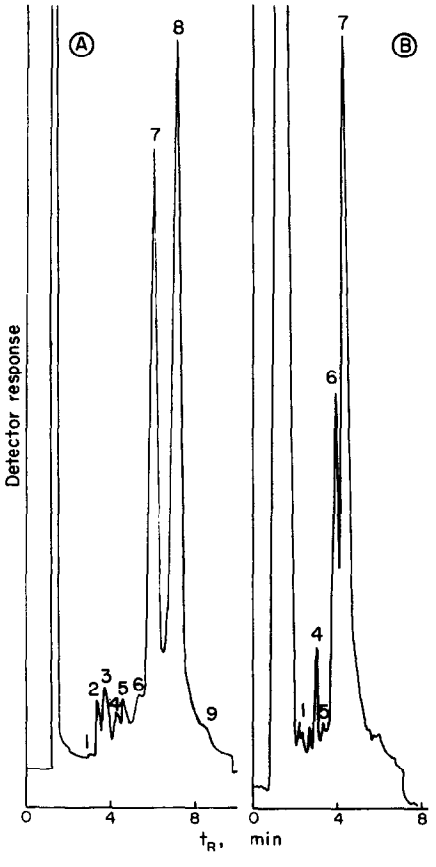

Fig. 1. Gas chromatograms of (A) 6β-hydroperoxyprogesterone, and (B) 6β-hydroperoxyandrostenedione on a 180 × 0.2 cm combination column, consisting of equal amounts of support, coated with 3% OV-1 and 3% OV-210. The peaks were eluted isothermally at 220°.

Twenty milligrams of non-radioactive 6β-OOH-progesterone and 10 μCi of C^{14}-labelled progesterone were dissolved together in 2.5 ml of dioxane. The S.A. of this solution was 995, 460 d.p.m./100 μl. To each of 10 Erlenmeyer flasks, containing 4 ml of adrenal fractions, 200 μl of the radioactive steroid solution was added. Bovine adrenal mitochondrial fractions were used for the first 5 flasks, and microsomal fractions for the remaining 5 flasks. The incubations were carried out under various conditions: in the presence or absence of PHMB, carbon monoxide, boiling of the incubation medium prior to addition of the steroids, in air, or in a closed high vacuum system under argon atmosphere. The incubations were terminated after 40 min by the addition of 10 ml of chilled acetone to each flask with vigorous shaking.

Incubation of 6β-hydroperoxyprogesterone with vegetative cultures of Rhizopus arrhizus. Cultures were obtained from spores of *Rhizopus arrhizus*, NRRL 6142, after a growth period of 24 h in two l. of corn steep medium containing 5% glucose, 2% peptone and 0.3% corn steep liquor. The mycelia were filtered over cheese cloth, rinsed with distilled water, resuspended in 0.5% NaCl with vigorous shaking, and filtered over cheese cloth. These washings were repeated twice. Five grams of the cells (wet weight) were suspended in 10.0 ml of 0.1 M phosphate buffer, pH 7.4. The incubation was started after the addition of a solution of 10 mg of 6β-hydroperoxyprogesterone in 1 ml of dimethylsulfoxide. After 16.5 h of shaking at 29°, the reaction was terminated by extraction with 10 ml of chilled methylisobutylketone. The reaction product obtained was purified by t.l.c., isolated, and analyzed by mass spectrometry.

RESULTS

Like their 17-hydroperoxypregnene analogues [23], 6β-hydroperoxyprogesterone and 6β-hydroperoxyandrostenedione undergo thermal decomposition to yield at least 7 products when subjected to gas chromatography (Fig. 1). Peaks no. 7 and 8 of chromatogram A were further identified by combined gas chromatography–mass spectrometry as respectively 6-ketoprogesterone, and 6β-hydroxyprogesterone. By the technique of simultaneous injection with known reference compounds, peak no. 2 in chromatogram A has been identified as progesterone. In analogous manner we have determined the identity of peaks no. 4, 6, and no. 7 in chromatogram B as respectively androstenedione, 6-ketoandrostenedione and 6β-hydroxyandrostenedione.

In the infrared spectra, the 6β-OOH group is characterized by 2 absorption bands: one in the region of 3300 and the other at 880 cm^{-1}. Upon dilution the 3300 cm^{-1} band shifted to a higher wave number, an indication that in the solid state the hydroperoxy group is not free, but hydrogen bonded.

Both 6β-hydroperoxides absorb in the U.V. region at 237 nm, which is the expected value for a 4-ene-3-

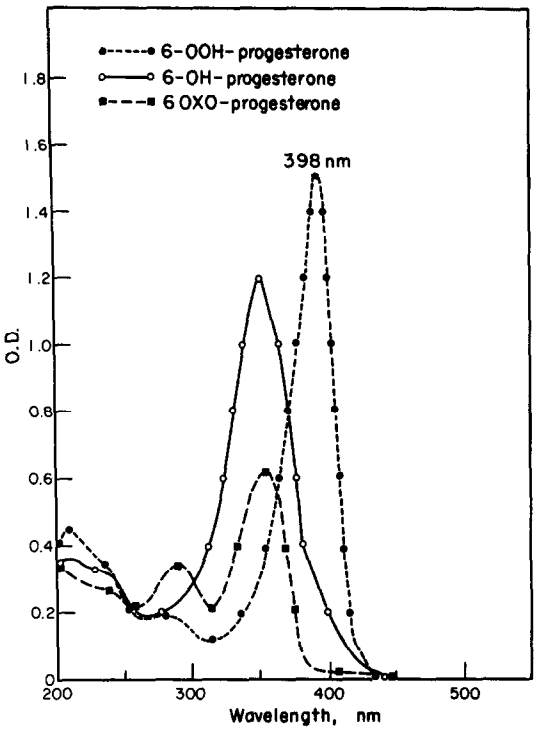

Fig. 2. Absorption spectra of 6β-hydroperoxyprogesterone, 6β-hydroxyprogesterone, and 6-keto-progesterone in concentrated sulfuric acid, after 2 h of reaction at 22° in the dark. The spectra were obtained by scanning the steroids from 200 nm to 450 nm at room temperature.

keto-steroid, bearing a β-substituent at position 6. More revealing are the U.V. spectra in concentrated sulfuric acid. In fact, both steroid hydroperoxides show after about 2 h at room temperature in concentrated sulfuric acid, an intense absorption maximum at 398 nm (Fig. 2). This band appears to be characteristic for a 6-hydroperoxy substituent, since it is absent in the sulfuric acid spectra of the corresponding 6-keto, 6-hydroxy, and also in the 6-nonsubstituted compounds, but is again present when 6β-hydroperoxycholestenone is treated in a similar manner with concentrated sulfuric acid. This property can therefore be used for the qualitative, and perhaps even quantitative analysis of 6-hydroperoxysteroids. The β-configuration of the hydroperoxy-substituent at position 6 was determined by chemical reduction to the known 6β-hydroxy steroid and further confirmed by proton magnetic resonance spectroscopy (Table 1).

It has been shown that in 4-ene-3-keto steroids bearing a substituent at C-6, there are 1, 3-interactions between the vinylic proton at C-4 and the α or β-proton at C-6 [24]. This interaction is greater when the hydrogen at C-6 is equatorial, or in other words, when the substituent has the β-configuration.

In the non-substituted progesterone molecule, the signal of the vinylic proton appears at 5.916 ppm. In 6β-OH-progesterone, there is a downfield shift of 0.042 ppm, and the signal appears now at 5.958 ppm. In the hydroperoxide which we have obtained, there is, with respect to progesterone, an even greater downfield shift of 0.134 ppm, with the vinylic proton signal now appearing at 6.050 ppm. We observe a similar downfield C-4 proton shift of 0.237 ppm with the 6-androstenedione hydroperoxide.

Analysis of the chemical shift of the 19-methyl protons is also revealing. In non-substituted progesterone, their signal appears at 1.400 ppm. In fact, only when the substituent at C-6 is in the β-configuration can we expect that the 19-methyl signal will be perturbed by long range effects. Indeed, in both hydroperoxides we observe a shift of the 19-methyl signal; upfield in the case of 6β-hydroperoxyprogesterone, and downfield in the case of 6β-hydroperoxyandrostenedione. There can be no doubt therefore, that the hydroperoxides which we have synthesized have both the β-configuration at C-6. It is interesting to note that when both hydrogen atoms at C-6 are replaced by oxygen, such as in 6-ketoprogesterone and 6-keto-androstenedione, the vinylic proton signal in both compounds now appear at the identical frequency of 6.133 ppm.

The chemical structure of the 6β-hydroperoxysteroids was further corroborated by subjecting them to mass spectrometry analysis. By carefully heating the solid introduction probe, we were able to obtain the molecular ions at *m/e* 318 and 346 (Fig. 3). Both hydroperoxides readily loose an oxygen atom, giving rise to intense peaks at respectively *m/e* 302 and *m/e* 330. In 4-ene-3-keto steroids bearing no substituents at positions 6, there is usually a significant ion at *m/e* 124. This is a ring-A fragment, generated by scission

Table 1. Comparative proton magnetic resonance data of the various 6-substituted progesterone and androstenedione compounds in deuterated chloroform using tetramethylsilane as an internal standard

Steroid	Chemical shift (ppm)				
	18-Me	19-Me	21-Me	C-4 (H)	Δ-C-4 (H)
Progesterone	0.700	1.400	2.166	5.916	0.000
6β-OH-Progesterone	0.716	1.416	2.175	5.958	+0.042
6β-OOH-Progesterone	0.675	1.366	2.166	6.050	+0.134
6-keto-Progesterone	0.673	1.140	2.140	6.133	—
Androstenedione	0.933	1.233	—	5.896	0.000
6β-OH-Androstenedione	0.991	1.466	—	6.050	+0.154
6β-OOH-Androstenedione	0.967	1.416	—	6.133	+0.237
6-keto-Androstenedione	0.913	1.173	—	6.133	—

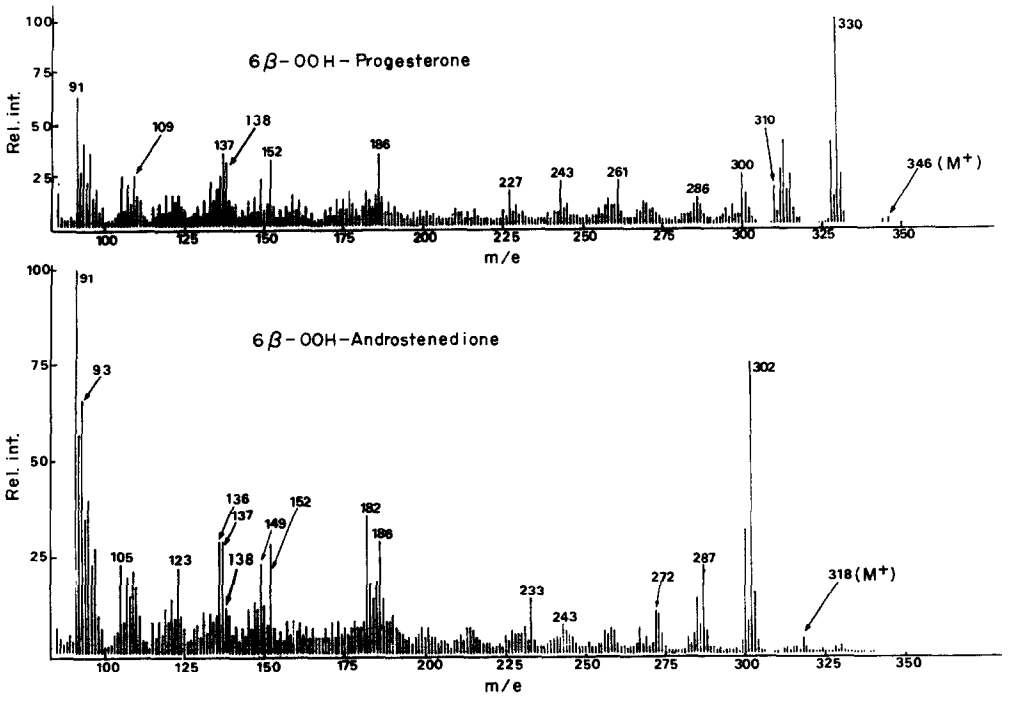

Fig. 3. Mass spectra of 6β-OOH-progesterone and 6β-OOH-androstenedione.

of the steroid molecule between C-9/C-10 and between C-6/C-7 [25]. In the case of our 6β-hydroperoxy compounds, there is no such significant ion. However, in both spectra, there now appears a new ion at *m/e* 138, due to formation of the same ring-A fragment, but now bearing an additional oxo-group at position 6. This ion is accompanied by a more intense, odd fragment ion at *m/e* 137, probably due to additional loss of a hydrogen. This type of fragmentation occurring in 6-keto-steroids has been reported previously by French authors [26]. Thus, mass spectrometry confirms not only that we have obtained a hydroperoxide, but also that this OOH-group is situated at C-6. In Fig. 4, the formation of 6β-hydroperoxyprogesterone and 6β-hydroperoxyandrostenedione at 4° as a function of time is shown when horseradish peroxidase was used as a catalyst in the presence of hydrogen peroxide. Starting from 5-pregnene-3, 20-dione and 5-androstene-3, 17-dione, there occurs a steady increase of the absorbance at 237 nm, indicative of the formation of a 6β-substituted 4-ene-3-ketone chromophore. The reaction

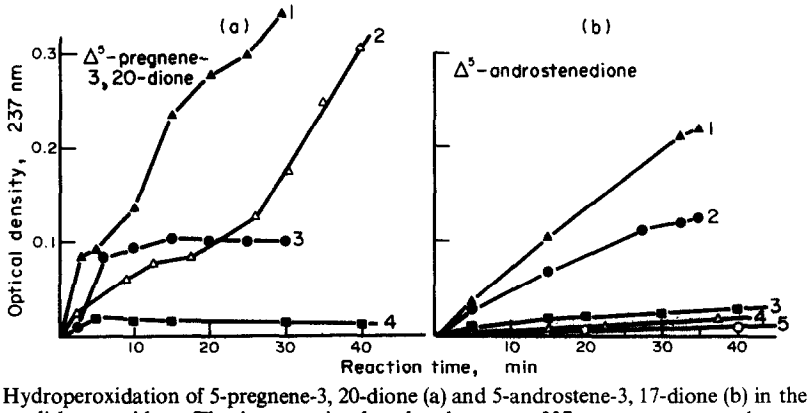

Fig. 4. Hydroperoxidation of 5-pregnene-3, 20-dione (a) and 5-androstene-3, 17-dione (b) in the presence of horseradish peroxidase. The increase in the absorbance at 237 nm was measured as a function of time at 4° using a Beckman model 24 double beam spectrophotometer. Sample and reference cuvettes contained the same ingredients, but no steroid was added in the reference cuvette, except in curve 5 in Fig. 4b. a: 1. 5-Pregnene-3, 20-dione(25 μg/25 μl dioxane) + H_2O_2(2 × 10^{-7}M) + 1650 μg peroxidase*. 2. 25 μg Steroid + H_2O_2(2 × 10^{-7}M) + 1100 μg peroxidase* + 600 μg catalase. 3. 25 μg Steroid + H_2O_2(2 × 10^{-7}M) + 550 μg peroxidase*. 4. 25 μg Steroid alone. b: 1. 5-Androstene-3, 17-dione(230 μg/25 μl dioxane) + H_2O_2(2 × 10^{-7}M) + 4 mg peroxidase*. 2. Steroid (110 μg/25 μl dioxane) + H_2O_2(2 × 10^{-7}M) + 4 mg peroxidase*. 3. Steroid (23 μg/25 μl dioxane) + H_2O_2(2 × 10^{-7}M) + 4 mg peroxidase*. 4. Steroid (23 μg/25 μl dioxane) + 4 ml 0.06 M Tris–HCl, pH 7.4. 5. As No. 3, with steroid also in the reference cuvette.

* In 4.0 ml of 0.06 M Tris-HCl, pH 7.4 buffer.

Table 2. Comparative hydroperoxidation of [4-^{14}C]-5-pregnene-3,20-dione in the presence of horseradish peroxidase

Reactants	% Yield				
	1	2	3*	4	5
Atmosphere	Air	Argon	Argon	Air	Air
H_2O_2	✓	✓	✓	✓	✓
Horseradish Peroxidase	✓	✓	Boiled	✓	✓
Catalase				✓	
PHMB					✓
Origin of t.l.c. spot	1.87	1.62	0.45	1.39	—
6β-OH-progesterone	2.67	0.15	1.20	3.32	0.67
6β-OOH-progesterone	10.98	3.41	1.68	21.77	3.21
6-keto-progesterone	14.98	21.75	10.45	19.06	20.27
Progesterone + 5-pregnene-3,20-dione	39.15	31.03	36.54	30.26	61.85
Sum of % yield of 6β-OH-, 6β-OOH-, and 6-keto-progesterone	28.63	25.31	13.33	45.54	24.15

* Control incubation. Incubation time 60 min at 0°C.

Each incubation flask contained 5.0 ml of 0.06 M Tris–HCl pH 7.4 buffer, horseradish peroxidase (28 mg/0.5 ml buffer), H_2O_2 (8×10^{-5} M) and a mixture of 1.66 mg of ^{14}C-labelled 5-pregnene-3,20-dione (specific activity, 55,500 d.p.m./mg) and 5.0 mg non-radioactive 5-pregnene-3,20-dione dissolved in 0.3 ml of dioxane. Catalase, 39 mg, was directly added to the medium (5.0 ml) of one of the flasks prior to the addition of the steroid. PHMB was added to one of the flasks to a final concentration of 4.0×10^{-3} M. Yields are expressed as a percentage of the radioactivity of the initial substrate.

clearly appears to be dependent upon the concentration of the enzyme (*cf.* curves 1 and 3 in Fig. 4a). We remark that these curves represent the formation of a mixture of both the 6β-hydroperoxy, and the 6β-hydroxysteroids; bearing in mind, however, that the enzyme system used does not contain any 6β-hydroxylase. Next, radioactive [4-^{14}C]-5-pregnene-3,20-dione was used as a substrate and the effect of certain parameters, such as the presence of oxygen and known hydroxylase inhibitors, studied. The formation of 3 products was quantitated (Table 2). In this table, the data of the control are listed in column 3. If we use the sum of the yields of the 3 metabolites, i.e. 6β-OOH, 6β-OH and 6-ketoprogesterone, as a parameter of the reaction, it is obvious that even in the absence of oxygen and active enzyme, there is still a relatively important formation of these metabolites (13.33%). Nevertheless, judged by this parameter, columns 1, 2, 4 and 5 all show much higher yields of the same metabolites. The high background data of the control can perhaps be explained by the inherent reactivity of the starting material which may have undergone some autoxidation during the course of the work-up, since it was technically not feasible to carry out the whole purification procedure in the complete absence of oxygen. However, when the reaction is followed *in situ* by spectrophotometry, the difference between the enzyme-catalyzed and the autoxidative reaction becomes more apparent (see Figs. 4a and 4b, *cf.* curves 1 and 4). It is evident from the data of Table 2 that much less 6β-hydroperoxide was formed when the incubation was conducted under argon. The 6β-hydroperoxides were also formed in much lesser amounts when the system was poisoned with PHMB. Catalase on the other hand, seems to enhance their formation instead of destroying the 6β-hydroperoxy-steroids; at least with the concentration we have used.

Table 3 shows the data we obtained when radioactive 5-pregnene-3,20-dione was incubated with bovine adrenal mitochondrial fraction under air or argon atmosphere. A significant decrease in the formation of the 6β-hydroperoxyprogesterone was observed in the incubation medium containing [4-^{14}C]-5-pregnene-3,20-dione and bovine adrenal mitochondria when PHMB was added, or the solution was bubbled with carbon monoxide, or the incubation was carried out under argon atmosphere. The formation of radioactive 6β-hydroperoxyprogesterone during incubations of [4-^{14}C]-progesterone with bovine adrenal mitochondrial or microsomal enzymes is shown in Table 4. The results indicated that more radioactive 6β-hydroperoxyprogesterone was trapped from [^{14}C]-labelled progesterone when microsomal enzymes were used in the incubation than with mitochondrial enzymes. PHMB significantly inhibited the formation of the [^{14}C]-labelled 6β-hydroperoxyprogesterone when either of the enzyme systems was used. However, carbon monoxide inhibition of the formation of the [^{14}C]-labelled 6β-hydroperoxyprogesterone could be observed only when mitochondria were used, and not in the case of microsomal enzymes. The necessity of oxygen is verified in either of the two enzyme systems as only a very small

Table 3. Comparative hydroperoxidation of [4-^{14}C]-5-pregnene-3, 20-dione in the presence of bovine adrenal mitochondria

Incubation No.	1	2	3	4
Steroid substrate	✓	✓	✓	✓
PHMB		✓		
CO			✓	
Argon atmosphere*				✓
Products		% Yield†		
Origin	30.2	13.9	13.9	6.1
6β-OH-progesterone	15.3	10.2	16.2	6.9
6β-OOH-progesterone	19.9	10.4	3.4	10.3
6-keto-progesterone	6.2	15.7	23.1	22.3
5-pregnene-3,20-dione + progesterone	4.8	15.4	15.1	40.4

* The incubation was conducted in a closed high vacuum system under argon atmosphere [16]. † Corrected for loss due to incomplete extraction and protein binding of the steroids.

Each incubation flask contained 10 ml of incubation buffer (100 parts of 2% bovine serum albumin in 0.15 M NaCl pH 7.4 buffer, 20 parts of 0.15 M of glycyl-glycine pH 7.4 buffer, and 1 parts of 0.11 M $MgCl_2$, and a NADPH generating system) and 68 mg of the bovine adrenal mitochondria. To each incubation flask was added 0.7 mg of [4-^{14}C]-5-pregnene-3,20-dione in 100 μl of dioxane (42,300 d.p.m.). Incubation flask No. 2 also contained PHMB (3.1×10^{-4} M). The incubations were terminated after 1 h at 22° by the addition of 10 ml of chilled methylisobutylketone with vigorous shaking.

amount of the radioactive 6β-hydroperoxyprogesterone could be trapped under argon atmosphere in a closed system.

Using the technique of reverse isotope dilution, the 6β-hydroperoxyprogesterone isolated from the incubation of [4-^{14}C]-progesterone with bovine adrenal microsomal enzymes was identified by repeated recrystallization from dioxane–water as shown in Table 5. Crystals after the third, fourth, fifth and sixth recrystallizations, and the mother liquid of the sixth recrystallization are shown to contain a specific activity of 402 ± 20 d.p.m./mg with 4.9% variation. *Rhizopus arrhizus* selectively hydroxylates progesterone at the 11α-position with formation of 6β-hydroxyprogesterone as a minor product [28]. In our observation, when 6β-hydroperoxyprogesterone was incubated with *Rhizopus arrhizus* only 6β, 11α-dihydroxyprogesterone was formed.

DISCUSSION

The 6-hydroperoxysteroids used in this study were chemically synthesized under conditions favouring a radical reaction. We could therefore expect the formation of both the 6α and 6β-epimers. Of these epimers, because of 1,3-diaxial interactions with the 19-methyl group, the 6α-epimer should be thermodynamically the more stable product. However, the only 6-hydroperoxide which we could isolate in crystalline form turned out to have the β-configuration. This can be readily determined from the NMR data (see Table 1). When the synthesis was carried out in a polar solvent system under slightly basic conditions in the presence of Cu^{2+} as a catalyst as described by the Roussel group [14], the only product which we could isolate was the 6-keto compound. This ketosteroid could only have been formed as the result of an internal rearrangement reaction of the corresponding hydroperoxide. Therefore, the initial products from the oxygenation reaction of 5-ene-3-ketosteroids are the 6-hydroperoxides, and the 6-keto and 6-hydroxy compounds must be viewed as secondary reaction products. Our failure to obtain the 6α-epimers may be due to this type of an internal hydroperoxide decomposition reaction, which appears to occur more readily when the OOH-substituent has the α-configuration.

We have shown in this study that incubation of 5-pregnene-3,20-dione with horseradish peroxidase, or with bovine adrenal fractions, leads in both cases to the formation of 6β-hydroperoxyprogesterone. In turn, the latter is further metabolized in the adrenals to 6-keto-, and 6β-hydroxyprogesterone. The crucial point to determine is whether the new 6β-hydroperoxy compounds, described for the first time in this paper, are formed exclusively by autoxidation, or whether they can also be construed as the products of a true enzymic reaction. Fig. 4 shows that their formation is not only time-dependent, but also dependent upon the concentration of the enzyme. Furthermore, under totally anaerobic conditions excluding any autoxidative reaction, the formation of 6β-hydroperoxy steroids, albeit in lesser quantities, could still be demonstrated. We are, therefore, tempted to assume that these 6β-hydroperoxy steroids can be formed from their 5-ene-3-keto steroid precursors, concurrently by an enzymic, and a non-enzymic reaction. This raises the question pertaining to the true status of the 6β-hydroxysteroids, which have been found in animal tissues. The problem is rendered more complex by the fact that a steroid-6β-hydroxylase enzyme, demonstrating a single catalytic activity, has never been obtained sofar, neither from animal, nor from microbiological sources; although its existence has been alluded to repeatedly in the literature. For instance, in the metabolism of deoxycholic acid, 6β-hydroxylation has been reported to constitute a minor pathway [29]. Voigt, *et al.*[30] have reported the presence in rat liver microsomes, of an active 6β-hydroxylase enzyme, which is oxygen- and NADPH-dependent. An important contribution to this field has been made by Toft[31]. He was able to show recently that in the metabolism of 6β-[^{2}H]-testosterone to 6β-hydroxytestosterone by rat liver microsomes, there was no deuterium label in the hydroxylated product. This result has been interpreted in terms of stereospecific loss of the label through the formation of a 3,5-dienol–enzyme complex, followed by the hydroxylation reaction itself, in accordance with the hypothesis of substitutions at activated carbon atoms, postulated earlier by Ringold[3]. How-

Table 4. Formation of radioactive 6β-hydroperoxyprogesterone during incubations of [4-^{14}C]-progesterone with bovine adrenal enzymes

	Microsomal enzymes					Mitochondrial enzymes				
Expt.	1	2	3	4	5§	6	7	8	9	10§
Steroid Substrate*	×	×	×	×	×	×	×	×	×	×
PHMB†		×					×			
NADPH	×	×	×	×	×	×	×	×	×	×
CO			×					×		
Air	×	×	×		×	×	×	×		×
Argon				×					×	
Boiling of enzymes					×					×
Product	% Yield‡									
Origin	0.72	0.56	0.36	0.53	0.85	1.19	0.34	1.20	0.97	0.50
6β-OH-4P	1.15	0.57	0.84	0.44	0.62	1.26	0.88	0.58		0.43
6β-OOH-4P	3.56	0.75	1.94	0.78	0.47	1.08	0.34	0.31	0.58	1.00
6-keto-4P	2.85	1.91	1.68	2.51	1.10	1.59	0.85	2.22	1.57	0.83
Progest.	50.11	55.46	57.57	64.42	59.98	57.90	61.61	43.89	62.41	58.32
Sum of 6β-OH, 6β-OOH-and 6-keto-progesterone	7.56	3.23	4.46	3.73	2.19	3.93	2.07	3.11		2.26

* 20 mg non radioactive 6β-OOH-progesterone mixed with [^{14}C]-progesterone (2.2×10^7 d.p.m.) in 2.5 ml dioxane. † Concentration: 6.5×10^{-3} M. ‡ Corrected for loss due to incomplete extraction and protein binding of the steroids. § Control incubations.

Bovine adrenal mitochondrial fraction, 43 mg in 4.0 ml of 0.06 M Tris–HCl buffer, pH 7.2, was added to each of the first 5 flasks, and microsomal fraction, 15 mg in 3.5 ml of the Tris–HCl buffer, was added to the remaining 5 flasks. Each flask contained 0.5 ml of NADPH generating system (4 mg of $NADP^+$, 6 mg of glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase and 1 mg of $MgCl_2$). Incubation No. 4 and No. 9 were done in a closed high vacuum system under argon atmosphere [16]. Incubations were terminated after 40 min at 22° by the addition of 10 ml of chilled acetone to each flask with vigorous shaking. The proteins were determined by Lowry method [27] using bovine serum albumin as reference.

ever, we would like to remark at this point that in all the above studies, the possible involvement of 5-ene-3-keto steroids has not been even considered.

As shown in this study, these β-γ-unsaturated ketones are, via the 6β-hydroperoxides, excellent substrates for the formation of 6β-hydroxysteroids. The 6β-hydroxylase system, which is involved in the bile acid metabolism of rat liver has been described to be cytochrome P-450 dependent [32]. In the present case, we can say that both 6β-hydroperoxyprogesterone and 6β-hydroperoxyandrostenedione readily bind to adrenal cytochrome P-450, as evidenced by the difference spectra they elicit. They are also rapidly metabolized to the corresponding 6β-hydroxy, and 6-ketosteroids. When radioactive progesterone was used as a substrate and incubated with bovine adrenal microsomes, a significant amount of radioactive 6β-hydroperoxyprogesterone could be trapped (see

Table 5. Identification of 6β-hydroperoxyprogesterone isolated from the incubation of [4-^{14}C]-progesterone with bovine adrenal microsomal enzymes by recrystallization from dioxane–water

	Specific activity (d.p.m./mg)	
Recrystallization No.	Crystals	Mother liquid
1.	1244	4818
2.	565	2909
3.	400*	1263
4.	414*	1151
5.	428*	520
6.	378*	389*
Mean	402	
S.D.	± 19.8	
% variation	± 4.92%	

* These are the data used in the calculation of the mean, standard deviation, and % of variation.

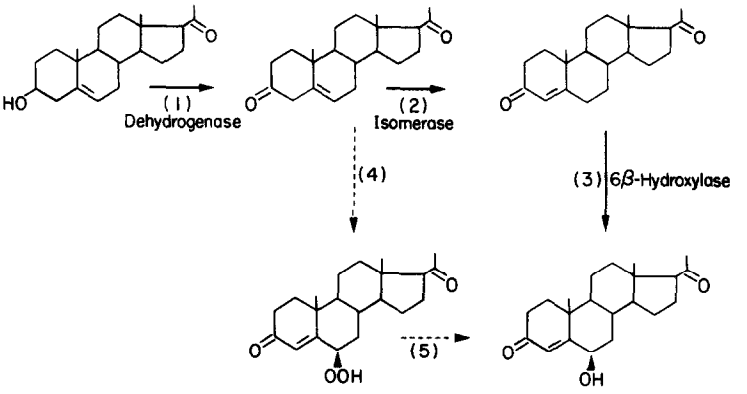

Fig. 5. Proposed mechanism for the formation of 6β-hydroxyprogesterone. The classical pathway of the formation of the steroid is shown by steps 1, 2 and 3. In our hypothesis, 6β-hydroxyprogesterone can be formed also by stereospecific hydroperoxidation at the 6β-position of the enolic form of progesterone, or of 5-pregnene-3-,20-dione as shown by steps 1 and 4, and by subsequent reduction of the 6β-hydroperoxyprogesterone (step 5).

Table 4). Recrystallisations to constant S.A. have proved that the trapped hydroperoxide was radioactively homogeneous (Table 5). The above results suggest that there may indeed exist a definite precursor-product relationship between 6β-hydroperoxy-, and 6β-hydroxyprogesterone, confirming our previous observations with 17α-hydroperoxypregnene compounds [33].

In conclusion, we would like to propose the hypothesis that 6β-hydroxyprogesterone can be formed in adrenal tissue by 2 different pathways: by the classical pathway (see Fig. 5, steps 1, 2 and 3), and via a 6β-hydroperoxide by a hitherto nondescribed pathway (steps 1, 4 and 5). Hydroperoxides are excellent initiators for radical chain reactions and, indeed, Fieser[10] has briefly alluded to the carcinogenic property of 6β-hydroperoxycholestenone. Therefore, a concerted effort to try to actually isolate these potentially damaging 6β-hydroperoxysteroids from animal tissues, would now be in order.

Acknowledgement—We thank the Medical Research Council of Canada for financial support, Mr. P. Masse and P. Rousseau for helpful technical assistance and Dr. P. Falardeau for his valuable comments.

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