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FORMATION OF 6β-HYDROPEROXYPROGESTERONE IN RAT LIVER MICROSOMES

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SUMMARY

To verify the concept that molecular oxygen can be enzymically introduced as such into the steroid nucleus, radioactive progesterone and also 5-pregnene-3,20-dione were incubated with microsomal preparations of rat liver. In both cases, a significant amount of radioactive 6β-hydroperoxyprogesterone was isolated, together with 6β-hydroxyprogesterone and 6-oxoprogesterone. Addition of *p*-hydroxymercuribenzoate or mercurichloride significantly inhibited the formation of the hydroperoxide, whereas potassium cyanide and carbon monoxide were only partially inhibitory. Addition of 6β-hydroperoxyprogesterone to cytochrome P-450 containing fractions of hepatic microsomes induced a Type I difference spectrum characterized by an absorption maximum at 392 nm, a minimum at 420 nm, and an isosbestic point at 407 nm. At 4°C, its apparent dissociation constant was found to be in the same order of magnitude as that of 6β-hydroxyprogesterone, namely 1.33 μM. A new pathway in rat liver for the formation of both 6β-hydroxyprogesterone and 6-oxoprogesterone via the 6β-hydroperoxide as a common precursor, is proposed.

INTRODUCTION

Apart from being the major organ in mammalians for the biosynthesis of cholesterol, the liver is essential for the catabolism of endogenous, as well as xenobiotic compounds. In the case of steroid hormones, they are inactivated in the liver by means of reactions which render them more hydrophilic, either by saturation of double bonds, or by the introduction of additional polar hydroxyl groups, and then excreting the transformed products in the form of water-soluble steroid conjugates. For instance, progesterone is converted for a major part in the liver to pregnanediol and excreted in the urine as its glucuronide [1]. One other metabolite of progesterone and of interest to us, is 6β-hydroxyprogesterone. Stereospecific hydroxylation at C-6 is thought of mainly as rendering the hormone biologically inactive. Thus, cortisol is one of the most potent natural corticosteroids known, whereas 6β-hydroxycortisol is inactive. In fact, the latter observation has formed the basis of the development of oral contraceptive steroids alkylated at C-6, e.g. 6-methyl-17α-acetoxyprogesterone; or chlorinated at C-6, such as chlormadinone acetate [2].

At present, the physiological importance of 6β-hydroxysteroids is not completely understood. In cases of Cushing's syndrome [3], or after administration of ACTH [4], 6β-hydroxycortisol appears to be the major unconjugated urinary metabolite of cortisol. In the urine of newborn infants, free 6β-hydroxycortisol also is present in relatively high concentrations [5]. Recently [6], we have demonstrated the existence of a pathway in the adrenal cortex leading to the formation of 6β-hydroxyprogesterone, via the previously unknown 6β-hydroperoxyprogesterone as an intermediate. Since the liver is known to contain much more of the "6β-hydroxylase" than the adrenal, we have looked into the possible occurrence of such an alternative pathway in the liver also, the results of which we report in this paper.

EXPERIMENTAL

Materials and methods

Progesterone, 6β-hydroxyprogesterone, 6-oxoprogesterone, and 5-pregnene-3,20-dione were obtained from Steraloids, Inc. Pregnenolone was from Organon, Holland. ^{14}C-labelled progesterone and pregnenolone were purchased from New England Nuclear Corp. 6β-Hydroperoxyprogesterone and ^{14}C-labelled 5-prenene-3,20-dione were synthesized as described earlier [6]. The specific activity of the latter compound used in this study was 165 μCi/mmol. All radioactive steroids used were purified to homogeneity by multiple thin-layer chromatography. Organic solvents were freshly distilled. Silica gel HF-254 and PF-254 were from Merck, A.G. Glucose-6-phosphate, G-6-P dehydrogenase and NADP were from Boehringer.

Gas chromatography

Analyses were performed with a Hewlett-Packard 7610A instrument. The U-type, 180 cm × 2 mm I.D. glass column was packed with 3% OV-1 and 3% OV-210 coated, 100–120 mesh Gaschrom Q particles, which were added simultaneously to both ends of the column. The peaks were eluted isothermally at 220°C with nitrogen as a carrier gas at a flow rate of 55 ml/min.

Infrared spectroscopy

The spectra were recorded with a Perkin–Elmer model 457 instrument, equipped with a beam condenser. Steroid/potassium bromide pellets were pressed using a weight ratio of approx. 1: 100.

Radioactivity measurements

Radioactivity was measured with a Beckman model LS 8000 counter. Aliquots of radioactive material were mixed in 15 ml of PCS-solution (Amersham Corp.) and counted for a minimum of 10 min. Counting efficiency for ^{14}C was found to be greater than 90%.

High-performance liquid chromatography

HPLC was carried out using a Whatman Partisil PAC-10 analytical column, an Altex model 110 solvent delivery pump, and an Altex-Hitachi variable wavelength detector. The steroid compounds were eluted isocratically at room temperature with a solvent mixture of ethanol–hexane (1:9, v/v) and with the detector set at 238 nm. The eluted peaks were collected manually.

Preparation of rat liver microsomes

Six adult, male Sprague–Dawley rats (300–350 g each) were killed, the livers immediately excised and immersed in ice cold 0.154 M KCl. All further manipulations were carried out at 4°C. The tissues were washed extensively with 0.154 M KCl, blotted on filter paper, and weighed. They were homogenized with 5 vol. (wt/vol.) of 0.25 M sucrose in a Waring blender for 2 min. The homogenate was filtered through cheese cloth and the filtrate centrifuged at 3020 *g* for 20 min to remove cell debris. The supernatant was carefully removed, filtered, and recentrifuged at 22,000 *g* for 20 min. This operation was repeated twice more. Thereafter, the supernatant was centrifuged at 105,000 *g* for 60 min in a Beckman ultracentrifuge at 4°C. The microsomal pellets were washed with 0.154 M KCl, homogenized, and recentrifuged at 105,000 *g* for 60 min. The latter process was repeated once again and the resulting washed microsomal pellets were used. Total protein content of the pellets was estimated using bovine serum albumin as standard [7].

Assays of cytochrome P-450

Cytochrome P-450 was assayed from the absorbance data of the carbon-monoxide–cytochrome P-450 complex, after reduction with sodium dithionite and using as extinction coefficient the value of 91/cm/mM [8].

Measurement of difference spectra

A typical experiment of the interaction of various pregnenes with rat liver cytochrome P-450 was performed as follows. The washed microsomal pellets were suspended in cold 0.1 M potassium phosphate buffer of pH 7.4, containing 20% glycerol and 0.2 mM EDTA. The suspension was homogenized in the cold using a Potter–Elvehjem apparatus. The difference spectra were recorded at 4°C using a Cary 19 spectrophotometer with automatic base line correction. Prior to the measurements, the presence of cytochrome P-450 was verified by reducing both the sample and reference cuvettes by dithionite, bubbling carbon monoxide in the sample cuvette for 1–2 min at the rate of approx. 70 bubbles/min, equilibrating for 5 min, and scanning the spectrum from 600–320 nm. The various steroids, dissolved in 20 μl of dioxane, were added in increasing concentrations to the sample cuvette, while an equal amount of neat dioxane was added to the reference cuvette. For these studies, the same batch of microsomes was used each time. The apparent spectral dissociation constants were then obtained by plotting the reciprocal values of ΔA versus concentrations, and extrapolation of the intercepts of the straight lines on the X-axis.

Incubations of [^{14}C]-pregnenes with rat liver microsomes

The washed microsomal pellets were homogenized at 4°C in 0.1 M of phosphate buffer of pH 7.4 containing 0.1 mM DTT, 0.1 mM EDTA, and 0.2% Tween-20, by means of a Potter–Elvehjem apparatus. The protein content of the homogenate was determined [7]. To each incubation flask were added: 4 mg microsomal protein (or as otherwise stated in the legends), and 0.5 ml of a NADPH-generating system consisting of 4 mg $NADP^+$, 6 mg glucose-6-phosphate, 2 units G-6-PD, and 1 mg $MgCl_2$ in the presence or absence of inhibitors. The total incubation volume was adjusted to 3 ml by adding phosphate buffer. The reaction was started by adding 0.75 μCi [^{14}C]-progesterone or [^{14}C]-5-pregnene-3,20-dione and 1.5 mg of unlabelled 6β-hydroperoxyprogesterone dissolved in 0.04 ml dimethylformamide. Incubations were terminated by adding 7 ml ice-cold dichloromethane and vigorous shaking. This extraction was repeated twice. The organic phases were pooled, washed, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness by a stream of nitrogen. The residue was dissolved in dioxane and further purified either by TLC, or HPLC.

RESULTS

Spectral studies

6β-Hydroperoxyprogesterone binds to cytochrome P-450$_{rlm}$ inducing a Type I spectral change characterized by a peak at 390 nm, a trough at 420 nm, and an isosbestic point at 407 nm (see Fig. 1). Since progesterone, 5-pregnene-3,20-dione, 6β-hydroxy-, and 6-oxoprogesterone all induce a Type I spectral change, their K_s values were measured (Table 1). 6β-Hydroxyprogesterone and 5-pregnene-3,20-dione had the same order of affinity, whereas progesterone and 6-oxoprogesterone show higher, identical K_s

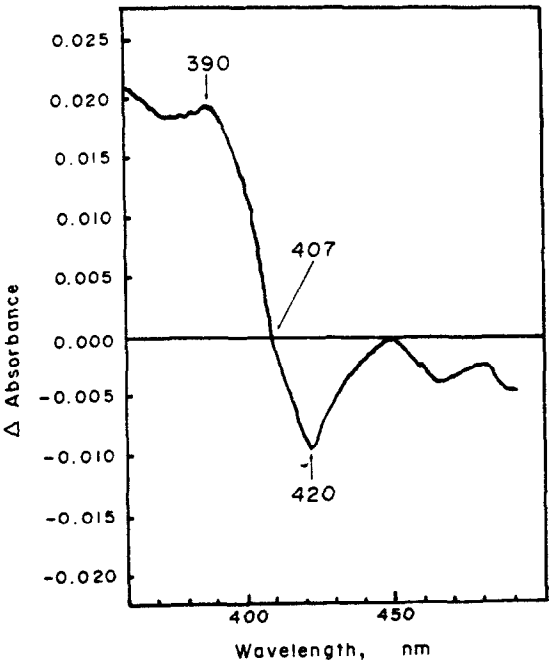

Fig. 1. Optical difference spectral effect induced by 6β-hydroperoxyprogesterone at 4°C on rat-liver microsomal cytochrome P-450. The steroid, dissolved in dioxane was added into the sample cuvette, and the same volume of neat dioxane was added into the reference cuvette. Both cuvettes contained 3 mg of protein/ml of microsomes.

values. Although our measurements were carried out at 4°C at which enzyme activity is negligible, these binding studies do not permit a clear distinction between 6β-hydroxy- and 6β-hydroperoxyprogesterone in their interaction with cytochrome P-450$_{rlm}$. We also found that progesterone was bound less tightly by a factor of 0.65 than its 5-ene isomer and that at 4°C, the latter compound does not undergo isomerization to the more stable 4-ene-3-one as shown by their different K_s values.

Metabolic studies

The conversion of [4-^{14}C]-progesterone into radioactive 6-oxygenated metabolites as a function of time is shown in Fig. 2. After 2 min, a plateau was reached for 6β-hydroperoxyprogesterone, whereas 6β-hydroxy- and 6-oxoprogesterone continued to be formed. Of these 3 steroids, the latter compound is thermodynamically the most stable, since in this steroid, the bulky 6β-axial substituent is replaced by a planar oxo group. Indeed, formation of the 6-oxo compound did not level off, even after 60 min of incubation. Moreover, in the first 2 min, the radioactive 6β-hydroperoxide was formed more rapidly than the other 2 oxygenated metabolites, suggesting the existence of a pathway in liver in which progesterone, via its 5-ene isomer, is first metabolized to the 6β-hydroperoxide. This compound can then, either spontaneously or enzymically, be converted into the more stable 6β-hydroxy and 6-oxo end products. The formation of [^{14}C]-5-pregnene-3,20-dione was detected by radio-TLC scanning (Fig. 3). In contrast to the other bands, band no. 6 corresponding to this radioactive steroid did not show u.v.-absorption. Other examples of similar 4-ene → 5-ene isomerisations have been reported previously [9, 10].

Table 1. Substrate-binding specificity of rat liver cytochrome P-450

Steroid substrate	Type of spectral change	K_s (μM)
6β-Hydroperoxyprogesterone	I	1.33
6β-Hydroxyprogesterone	I	1.28
Progesterone	I	2.27
5-Pregnene-3,20-dione	I	1.49
6-Oxoprogesterone	I	2.27

Effect of inhibitors

The effect of various inhibitors on the formation of 6-oxygenated progesterone metabolites is summarized in Table 2. Compared to the assigned control value of 100%, only 1.25% of radioactive hydroperoxide was obtained when the incubation was carried out under nitrogen atmosphere in the absence of air. Lower yields of the hydroperoxide were also obtained when NADPH was omitted from the incubation medium (19% of control). The well-known cytochrome P-450 inhibitors, potassium cyanide and carbon monoxide only partially inhibited formation of the hydroperox-

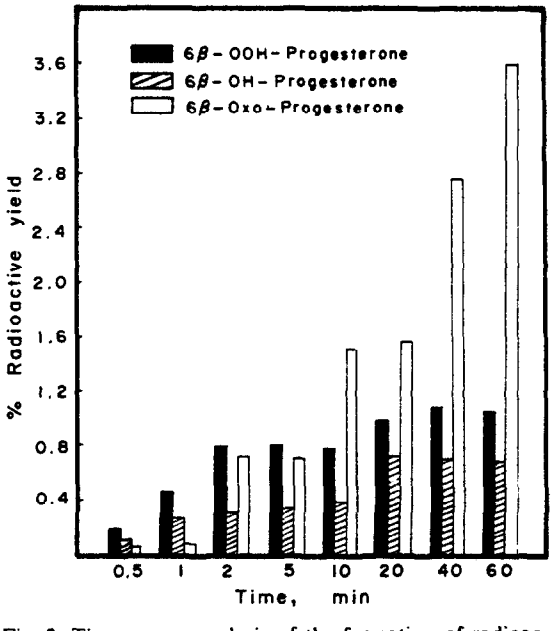

Fig. 2. Time course analysis of the formation of radioactive 6-oxygenated progesterone metabolites. A mixture of 0.75 μCi of [4-^{14}C]-progesterone and 1.5 mg of non-labeled 6β-hydroperoxyprogesterone was incubated at 37 C with rat liver microsomes in 3.0 ml of phosphate buffer, pH 7.2. Protein concentration was 5 mg/ml. After extraction, the steroid metabolites were separated by thin-layer and high-performance liquid chromatography, prior to counting.

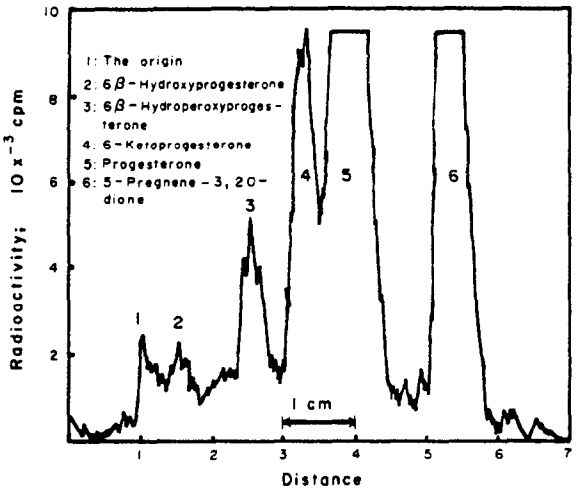

Fig. 3. Radiochromatogram of [^{14}C]-labeled steroids extracted from incubation of [^{14}C]-progesterone with rat liver microsomes. The extract was applied on a 5 × 20 cm glass plate coated with a 0.1 mm thick layer of Silica gel HF_{254} and irrigated with a toluene–ethylacetate (4:1, v/v) mixture as the mobile phase. The plate was scanned with a Packard model 7201 instrument set at 0.9 kVolt.

ide (31 and 38% resp.). In contrast, *p*-hydroxymercuribenzoate, a thiol-blocking agent, inhibited strongly (87%). The total yield of radioactive 6-oxygenated steroids obtained under anaerobic conditions was 1.37%. This amount may be due to autoxidation of the remaining substrate after termination of the incubation, and accumulating during the course of the work-up.

Effect of protein concentration

The effect of microsomal protein concentration on the formation of radioactive 6-oxygenated progesterones is shown in Table 3. The values obtained with heat-inactivated samples may again be attributed to auto-oxidation. However, taking these non-enzymic processes into account, the corrected values clearly show for all 3 oxygenated metabolites a significant, protein concentration-dependent increase.

High pressure liquid chromatography

Steroid hydroperoxides are known to readily undergo light-, temperature-, and solvent-dependent rearrangement reactions. Purifications on silica gel TLC-plates, even when carried out in the cold room and in the dark, still give rise to decomposition products. For this reason, we have resorted to HPLC analysis also. 6β-Hydroperoxyprogesterone was purified on a cyano-, and aminosilyl prepacked column with a mixture of hexane–ethanol as the eluant (see Fig. 4). By avoiding the use of an aqueous solvent system, the chemical structure of the otherwise labile hydroperoxide moiety can be preserved. This was established by collecting the hydroperoxide contain-

Table 2. Effect of inhibitors on the formation of 6-oxygenated 4-pregnene-3,20-diones from [4-^{14}C]-progesterone incubated for 20 min at 37°C with rat liver microsomes

Inhibitory condition	Total radioactive yield in %* (% yield relative to control in parenthesis)			
	6β-OOH-4P	6β-OH-4P	6-oxo-4P	Sum of 3
None (control)	4.00 (100)	4.59 (100)	5.79 (100)	14.38 (10))
N_2 atmosphere	0.05 (1.25)	0.58 (12.6)	0.74 (12.8)	1.37 (9.5)
0.1 M KCN	2.77 (69.2)	2.56 (55.8)	2.21 (38.2)	7.54 (52.4)
4 mM pHMB	0.51 (12.8)	2.47 (53.8)	1.20 (20.7)	4.18 (29.1)
No NADPH	0.77 (19.2)	1.71 (37.3)	2.43 (41.2)	4.91 (34.1)
Carbon monoxide	2.47 (61.8)	2.96 (64.5)	2.20 (37.9)	7.63 (53.1)

* Values were subtracted from the boiled incubation sample.
Incubations were conducted in duplicate.

Table 3. Effect of microsomal protein concentration on formation of 6-oxygenated 4-pregnene-3,20-diones from a 20 min incubation at 37°C of [4-^{14}C]-progesterone

Protein concentration (mg/ml)	% Total radioactivity recovered (values corrected for autoxidation in brackets)			
	6β-OOH-4P	6β-OH-4P	6-oxo-4P	Sum of 3
2.0 (boiled)	1.06	0.91	1.32	3.29
0.1	1.0 (0.04)	1.23 (0.32)	1.32 (0.0)	3.65 (0.36)
0.2	1.17 (0.11)	1.24 (0.33)	1.36 (0.04)	3.77 (0.48)
0.5	1.32 (0.26)	1.49 (0.58)	1.54 (0.22)	4.35 (1.06)
1.0	1.36 (0.30)	1.67 (0.76)	1.81 (0.49)	4.84 (1.55)
2.0	2.23 (1.17)	2.17 (1.26)	3.01 (1.69)	7.41 (4.12)
5.0	3.86 (2.80)	3.80 (2.89)	3.03 (1.71)	10.69 (7.40)

Values are mean of duplicate incubations.

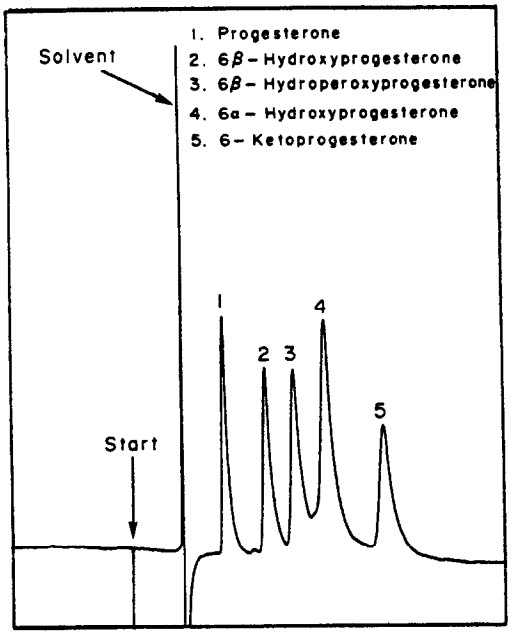

Fig. 4. Resolution of 6-oxygenated 4-pregnene-3,20-diones by high performance liquid chromatography. Steroid samples were injected directly into a Whatman Partisil-10 PAC analytical column and eluted with a mixture of ethanol–hexane (1:9, v/v) Flowrate: 0.9 ml/min; detection: 238 nm; sensitivity: 0.05 A.f.s. Fractions were collected manually and counted for radioactivity.

Table 4. Identification of radioactive 6β-hydroperoxyprogesterone isolated from rat liver microsomes by recrystallizations from dioxane–water

Recrystallization number	Specific activity (c.p.m./mg)	
	Crystals	Mother liquid
1	70.63	394.17
2	59.03	264.53
3	46.42	134.09
4	46.92	46.75

ing fractions and then determining its characteristic i.r.-spectrum ($\bar{\nu}_{OOH} = 3300\,cm^{-1}$) and melting point (164–168°C) of the carefully dried residue. Also, when the collected hydroperoxide was reduced with potassium iodide in dilute acetic acid, the product obtained was identical in all aspects with reference 6β-hydroxyprogesterone. At present, HPLC appears to be the only suitable method for the rapid isolation and purification of labile steroid hydroperoxides.

Proof of radioactive homogeneity of the isolated 6β-hydroperoxyprogesterone

The radioactive peak, co-eluting in HPLC with authentic reference material was co-crystallized with 22.67 mg of unlabelled 6β-hydroperoxyprogesterone. After 4 recrystallizations, the crystals and the supernatant had the same specific activities (see Table 4).

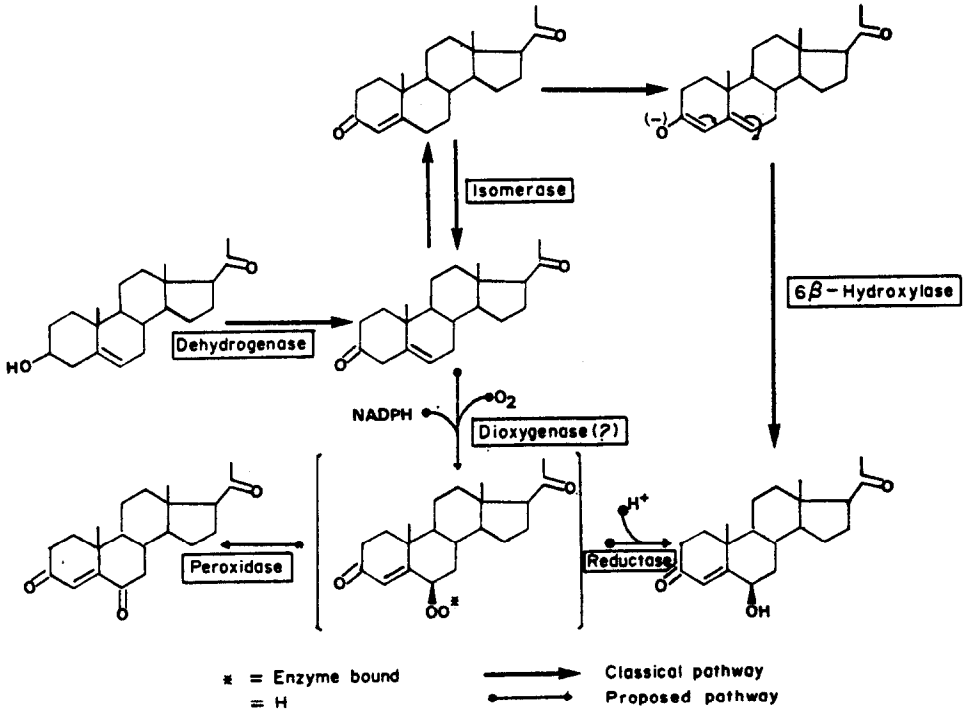

Fig. 5. Alternative pathways for the formation of 6β-hydroxyprogesterone. According to the classical pathway (heavy arrows) progesterone, via the 3,5-dienol, is stereospecifically hydroxylated by a 6β-hydroxylase. A second pathway (thin arrows) proceeds via the 5-ene-3-one and a transient, labile intermediate that collapses into the 6β-hydroxy and 6-oxo stable endproducts.

DISCUSSION

The results show that progesterone undergoes oxidation at C-6 by rat-liver microsomal preparations to yield the corresponding 6β-hydroperoxy-, 6β-hydroxy-, and 6-oxo compounds. This reaction requires not only molecular oxygen and NADPH, but is also dependent on the concentration of the microsomal proteins. Moreover, the oxidation is inhibited significantly by pHMB, but only partially by potassium cyanide and carbon monoxide. Although some autoxidation due to the work-up cannot technically be prevented, we believe that the major part of the 6β-hydroperoxyprogesterone isolated is of enzymic origin. Whether we are dealing with a cytochrome P-450 type enzyme is, however, questionable in view of the slight inhibition exerted by potassium cyanide and carbon monoxide. Classically, stereospecific hydroxylation at C-6 of progesterone is depicted as proceeding via the 3,5-dienol, catalyzed by a cytochrome P-450 type monooxygenase, i.e. 6β-hydroxylase [11, 12]. Concomittant formation of the 3 oxygenated progesterone metabolites at C-6 as shown in this work can, however, also be explained by a pathway in which progesterone, via its deconjugated 5-ene-3-one isomer, is first oxygenated to a reactive peroxy intermediate, which then, either spontaneously or enzymically, is converted into the stable 6β-hydroxy and 6-oxo end products (Fig. 5). The formation of these 3 oxidation products of progesterone is not limited to liver microsomes; similar results have been obtained also with bovine adrenal gland preparations [6]. The presence of steroid hydroperoxides in animal tissues, however fleeting it may be, is not devoid of physiological importance. The hydroperoxide group can generate radicals and initiate a pathological chain reaction analogous to that caused by lipid hydroperoxides [13, 14]. Further studies on the biological effects of steroid hydroperoxides are necessary.

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