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Properties of Hydroxylase Systems in the Human Fetal Liver Active on Free and Sulfoconjugated Steroids†

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ABSTRACT: The substrate specificity of the steroid sulfate-hydroxylating activity in microsomes from human fetal liver has been investigated. Twelve different C_{18}, C_{19}, C_{21}, and C_{27} steroid sulfates and the corresponding free steroids were used as substrates. The introduction of a sulfate group on the steroid substrate was found to have two principal effects. (1) The hydrophilic sulfate group directs the steroid molecule so that it only interacts with the active site of cytochrome P-450 with its non-sulfurylated, hydrophobic end. (2) The sulfate group interacts with the enzyme surface resulting in exposure of a slightly different part of the hydrophobic end of the substrate to the active site of cytochrome P-450 than when the same end of the free steroid is exposed to the active site of the enzyme. As a consequence of these two effects of the sulfate group, the "steroid sulfate pathway" of steroid hydroxylations generally differs considerably from the "free steroid pathway," both from a qualitative and a quantitative aspect. This difference was found to be most pronounced with estrogens: whereas estradiol was not hydroxylated by human fetal liver microsomal preparations, estradiol 3-sulfate was both 15α- and 16α-hydroxylated. Thus, for certain steroids, sulfurylation is a prerequisite for further metabolism by microsomal hydroxylase systems. These results indicate the presence in human fetal liver microsomes of a multipotent, highly unspecific, hydrophobic "bulk" of cytochrome P-450. The existence of this hydroxylase system which efficiently hydroxylates steroid sulfates is probably of great physiological importance as a detoxifying mechanism in the human fetus.

The human fetal tissues have a high sulfurylase but very low sulfatase activity (Diczfalusy, 1969). Consequently, sulfoconjugated steroid hormones are present in high concentrations in fetal tissues (Huhtaniemi *et al.*, 1970). During pregnancy, steroid sulfates are known to be important precursors of other steroid hormones (Diczfalusy, 1969) and it has been suggested that the sulfoconjugated steroids may be directly metabolized without prior cleavage of the sulfate group (Huhtaniemi, 1974).

In view of these considerations and of our recent finding of a specific steroid sulfate-hydroxylating enzyme system in female rat liver (Gustafsson and Ingelman-Sundberg, 1974), we have undertaken a study of the substrate specificity of the human fetal liver microsomal enzyme system that catalyzes the hydroxylation of steroid sulfates. The results presented in this paper indicate the existence of a steroid sulfate-hydroxylating species of cytochrome P-450 in human fetal liver which is completely different from the corresponding species in rat liver.

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Materials and Methods

Steroids. Radioactive and nonradioactive steroids were synthesized or obtained as described in Table I. The sulfate conjugates of the steroids were synthesized essentially according to Mumma *et al.* (1969), purified as described before (Gustafsson and Ingelman-Sundberg, 1974), and recrystallized from acetone or methanol. Phenolic sulfate esters were liable to hydrolyze spontaneously and were incubated immediately after recrystallization. Reference steroids utilized in the identifications were obtained from sources specified previously (Berg and Gustafsson, 1973; Einarsson *et al.*, 1973a,c).

Biological Material. Human fetuses (see Table II) (22) were obtained at legal abortion for socio-medical reasons. The abortions were performed *via* hysterotomy. Only fetuses from healthy women were included in the investigation. The fetuses were immediately transported to the laboratory and preparation of the microsomal fraction of the liver was started within 45 min after abortion.

Experimental Conditions. Liver homogenates, 20% (w/v), were prepared either in a modified Bucher medium (Bergström and Gloor, 1955), pH 7.4, or in 0.25 M sucrose, with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 9000 *g* for 10 min and the resulting supernatant was centrifuged at 105,000*g* for 70 min. The protein concentration of the microsomal fraction was determined

Table I: Data on Labeled and Unlabeled Steroid Substrates Used in the Present Investigation.

Steroid	Type of Label	Specific Activity and Source of of Labeled Steroid	Source of Unlabeled Steroid	Summary of Synthetic Procedure
17β-Hydroxy-4-androsten-3-one	[4-^{14}C]	57.5 Ci/mol, NEN[a]	Upjohn Co.[b]	
(testosterone)	[4-^{14}C]	57.5 Ci/mol		From testosterone according to Mumma *et al.* (1969)
17β-Hydroxy-5α-androstan-3-one (5α-dihydrotestosterone)	[4-^{14}C]	56.1 Ci/mol, NEN	Sigma[c]	
5α-Dihydrotestosterone 17-sulfate	[4-^{14}C]	56.1 Ci/mol		From dihydrotestosterone according to Mumma *et al.* (1969)
5α-Androstane-3α,17β-diol	[4-^{14}C]	57.5 Ci/mol	Sigma	Labeled steroid from 4-[4-^{14}C]-androstene-3,17-dione[d] according to Berg and Gustafsson (1973)
5α-Androstane-3α,17β-diol	[1,2-^{3}H]	45.2 Ci/mmol, NEN	Sigma	
5α-Androstane-3α,17β-diol 3,17-disulfate	[1,2-^{3}H]	45.2 Ci/mmol		From 5α-androstane-3α,17β-diol according to Mumma *et al.* (1969)
5α-Androstane-3β,17β-diol[e] 17-sulfate	[4-^{14}C]	56.1 Ci/mol		From 5α-dihydrotestosterone 17-sulfate by $NaBH_4$ reduction (Gustafsson and Sjövall, 1968b)
3α-Hydroxy-5α-androstan-17-one (androsterone)	[1,2-^{3}H]	53.0 Ci/mmol, NEN	Ikapharm[f]	
Androsterone 3-sulfate	[1,2-^{3}H]	53.0 Ci/mmol		From androsterone according to Mumma *et al.* (1969)
3β-Hydroxy-5-androsten-17-one (dehydroepiandrosterone)	[4-^{14}C]	57.1 Ci/mol, NEN	Ikapharm	
Dehydroepiandrosterone 3-sulfate	[4-^{14}C]	57.1 Ci/mol		From dehydroepiandrosterone according to Mumma *et al.* (1969)
3-Hydroxy-1,3,5(10)-estratrien-17-one (estrone)	[4-^{14}C]	62.1 Ci/mol, NEN	Sigma	
Estrone 3-sulfate	[4-^{14}C]	62.1 Ci/mol		From estrone according to Mumma *et al.* (1969)
1,3,5(10)-Estratriene-3,17β-diol (estradiol)	[4-^{14}C]	52.1 Ci/mol, NEN	Sigma	
Estradiol 17-sulfate	[4-^{14}C]	52.1 Ci/mol		From estradiol according to Mumma *et al.* (1969)
Estradiol 3-sulfate	[4-^{14}C]	52.1 Ci/mol		From estrone 3-sulfate by $NaBH_4$ reduction (Gustafsson and Sjövall, 1968b)
3β-Hydroxy-5-pregnen-20-one	[4-^{14}C]	56.7 Ci/mol, RCA[g]	South-eastern[h]	
Pregnenolone 3-sulfate	[4-^{14}C]	56.7 Ci/mol		From pregnenolone according to Mumma *et al.* (1969)
21-Hydroxy-4-pregnene-3,20-dione (deoxycorticosterone)	[1,2-^{3}H]	55.0 Ci/mmol, RCA	Upjohn Co.	
Deoxycorticosterone 21-sulfate	[1,2-^{3}H]	55.0 Ci/mmol		From deoxycorticosterone according to Mumma *et al.* (1969)
5-Cholesten-3β-ol (cholesterol)	[4-^{14}C]	55.0 Ci/mol, RCA	Aptekarnes[i]	
Cholesteryl 3-sulfate	[4-^{14}C]	55.0 Ci/mol		From cholesterol according to Mumma *et al.* (1969)

[a] NEN, New England Nuclear Corp., Boston, Mass. [b] Upjohn Co., Kalamazoo, Mich. [c] Sigma Chemical Co., St. Louis, Mo. [d] 4-[4-^{14}C]Androstene-3,17-dione (specific activity, 57.5 Ci/mol) was purchased from NEN. [e] Schering AG, Berlin, Germany. [f] Ikapharm, Ramat-Gan, Israel. [g] RCA, Radiochemical Centre, Amersham, England. [h] Southeastern Biochemicals, Morristown, Tenn. [i] Apotekarnes Droghandel AB, Stockholm, Sweden.

according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Incubations with free steroids and corresponding sulfate esters were performed using the same preparation of liver microsomes. Usually 700 nmol and 400,000 dpm of each substrate was used. Free steroids were dissolved in 100 μl of

Table II: Characteristics of Fetuses Used in the Present Investigation.

Fetus No.	Gestational Age (weeks)	Crown-Rump Length (cm)	Sex	Liver Weight (g)
1	16		M	4.0
2	20		M	7.0
3	10		F	0.2
4	13	6.8		0.6
5	14	7.8		1.1
6	24	21.0	M	22.0
7	22	18.5	F	14
8	18	13.0	M	5.7
9	19	14.0	M	7.8
10	14	8.2	F	4.2
11	12		F	1.0
12	24	22.0	M	24
13	15	10.4	M	3.4
14	14	8.8	M	2.4
15	20	16.0	F	10.8
16	15	10.4	M	3.1
17	16	11.2	M	4.2
18	16	10.0	F	3.5
19	15	9.7	F	2.5
20	18	12.3	M	5.5
21	17	10.4	M	5.6
22	16	9.4	M	2.2

acetone and steroid sulfates in 100 μl of distilled water. The incubations were started by adding the substrate solution to a mixture of microsomal protein from 0.3 to 1.0 g of liver (corresponding to about 2.5–8.5 mg of microsomal protein), 0.03 μmol of $MnCl_2$, 4 μmol of $NADP^+$, 12.5 μmol of isocitrate, and 0.36 U of isocitrate dehydrogenase in a total volume of 4 ml of Bucher medium that had been preincubated at 37° for 5 min. The incubations were carried out for 10–30 min at 37° and terminated by the addition of 10 ml of chloroform–methanol, 2:1 (v/v). The incubation conditions chosen for each substrate were designed to give conversions linear with respect to time and enzyme concentration. In all experiments control incubations were performed using the same conditions as described above except that 10 ml of chloroform–methanol, 2:1 (v/v), was added to the incubation mixture immediately after addition of the substrate.

Extraction and Purification of Metabolites Formed. From incubation mixtures containing free steroids with the exception of estrogens, the precipitate was filtered off and 0.2 volume of an aqueous solution of sodium chloride (0.9%, w/v) was added. The chloroform phase was collected and the solvent was evaporated. The residue was subjected to thin-layer chromatography using the solvent systems given in Table III. Thin-layer plates with extracts from incubations with ^{3}H-labeled steroids were scanned for radioactivity using a Berthold thin-layer scanner, Model II (Berthold, Wildbad, Germany). Thin-layer plates with extracts from incubations with ^{14}C-labeled steroids were subjected to radioautography with an exposure time of 7 days. Radioactive zones were scraped off the thin-layer plates, extracted with methanol, measured for radioactivity in a Packard liquid scintillation spectrometer, Model 4322, using Instagel^R as scintillator liquid (^{3}H-labeled metabolites were quantitated directly from the scanning chromatograms), and analyzed

Table III: Solvents Systems Used for Thin-Layer Chromatography of Free (Including Solvolyzed) Steroid Metabolites.

Metabolites of	Solvent System	No. of Developments
Testosterone 5α-Dihydrotestosterone Dehydroepiandrosterone Pregnenolone	Chloroform–ethyl acetate, 4:1 (v/v)	2
5α-Androstane-3α,17β-diol	Cyclohexane–ethyl acetate, 2:3 (v/v)	3
Estradiol	Ethyl acetate–cyclohexane–ethanol, 9:10:1 (v/v)	1
Deoxycorticosterone	Ethyl acetate–*n*-hexane–acetic acid, 75:20:5 (v/v)	1
Cholesterol	Benzene–ethyl acetate, 1:1 (v/v)	1

by gas chromatography–mass spectrometry.

Incubation mixtures containing estrogens were diluted with 100 ml of chloroform–methanol, 2:1 (v/v), and allowed to stand in an ultrasonic water bath for 20 min. After centrifugation at 5000*g* for 30 min the supernatant was decanted and evaporated to dryness. The residue was applied on a thin-layer plate and analyzed as described above.

Incubation mixtures containing steroid sulfates were extracted according to the same procedure as described for estrogens. The residue obtained after evaporation of the 5000*g* supernatant was chromatographed on a column of Sephadex LH-20 with chloroform–methanol, 1:1 (v/v), 0.01 M with respect to NaCl, as solvent system. When a steroid monosulfate had been incubated, less than 1% of the total radioactivity was eluted in the free steroid fraction and when a steroid disulfate had been incubated, less than 1% was eluted in the combined free steroid and monosulfate fractions. The mono- or disulfate fraction—depending on the nature of the substrate—was evaporated to dryness and the residue was solvolyzed in acidified ethyl acetate. The liberated steroids were subjected to thin-layer chromatography and analyzed as described above.

Gas Chromatography–Mass Spectrometry Analysis. Steroids recovered from radioactive thin-layer chromatographic zones were silylated and analyzed by gas chromatography–mass spectrometry employing an LKB 9000 instrument; 1.5% SE-30 was used as stationary phase. Retention times (t_R) were calculated relative to 5α-cholestane. Mass spectra were recorded on magnetic tape using the incremental mode of operation and were treated in an IBM 1800 computer (Reimendal and Sjövall, 1972). A compound was considered identified if it had the same mass spectrum and gas–liquid chromatographic behavior as the reference compound. Thin-layer chromatographic zones with more than one metabolite were quantitated by gas–liquid chromatography, using a Pye gas chromatograph, Model 64 (WG Pye & Co., Ltd., Cambridge, England), with 1.5% SE-30 as the stationary phase.

Results

The hydroxylated metabolites formed after incubation of various free steroids and corresponding sulfate esters with

Table IV: Human Fetal Liver Microsomal Hydroxylase Activities on Free and Sulfoconjugated Steroids and Gas Chromatographic-Mass Spectrometric Characteristics of Hydroxylated Metabolites.[a]

Substrate	Incubation Performed on Fetus No.	Hydroxylated Metabolites Isolated (in the Case of Sulfoconjugated Metabolites, the Sulfate Group is not indicated)	Specific Enzyme Activity (nmol/mg of protein per min)	t_R (SE-30)	Molecular Ion	Base Peak	Other Prominent Peaks	Ref
Testosterone	5,16[b]	1β-Hydroxytestosterone	0.068	0.91	448	332	129,242, 433	Lisboa and Gustafsson, 1968a
		2α-Hydroxytestosterone	0.037	0.90	448	433		
		2β-Hydroxytestosterone	0.033	1.06	448	433		Lisboa *et al.*, 1968
		6β-Hydroxytestosterone	0.115	0.79	448	392	129,433, 448	Lisboa *et al.*, 1968
		18-Hydroxytestosterone	0.069	0.98	448	129	191,217, 268,358	Lisboa and Gustafsson, 1969
Testosterone sulfate	5, 16[b]	2α-Hydroxytestosterone	0.007	0.90	448	433		
		2β-Hydroxytestosterone	0.432	1.06	448	433		Lisboa *et al.*, 1968
5α-Dihydrotestosterone	11,12[b]	18-Hydroxy-5α-dihydrotestosterone	0.074	0.81	450	360	129,191, 217,270, 319	Gustafsson and Lisboa, 1969
5α-Dihydrotestosterone sulfate	11,12[b]	4-Hydroxy-5α-dihydrotestosterone	0.252	0.95	450	435	129,253	
5α-Androstane-3α,17β-diol	7	5α-Androstane-3,4,17β-triol	0.088	0.87	524	215	255,393, 395,434	
		5α-Androstane-3α,6α,17β-triol	0.534	0.77	524	344	129,254, 434	Gustafsson *et al.*, 1968b
		5α-Androstane-3α,15α,17β-triol	0.054	0.68	524	217	191	Gustafsson and Sjövall, 1968b
		5α-Androstane-3α,16α,17β-triol	0.559	0.91	524	191	434,524	Gustafsson *et al.*, 1968b
		5α-Androstane-3α,17β,18-triol	0.169	0.73	524	217	191,344, 419,434	Gustafsson and Lisboa, 1969
5α-Androstane-3β,17β-diol 17-sulfate	7	5α-Androstane-2α,3β,17β-triol	0.203	1.08	524	255	129,142, 143,509	Einarsson *et al.*, 1973c
		5α-Androstane-3,4,17β-triol	0.104	0.87	524	215	255,393, 395,434	
5α-Androstane-3α,17β-diol disulfate	1,2[b]	None	< 0.001					
Androsterone	6	6-Hydroxyandrosterone	0.033	0.60	450	271	270,360, 450	
		16α-Hydroxyandrosterone	0.615	0.65	450	216	106,117, 306	Gustafsson and Lisboa, 1970a
		18-Hydroxyandrosterone	0.312	0.70	450	420	103,169 360	Gustafsson and Lisboa, 1970b
Androsterone sulfate	6	18-Hydroxyandrosterone	0.648	0.70	450	420	103,169, 228,360	Gustafsson and Lisboa, 1970b

(Continued)

Table IV: *(Continued)*

Substrate	Incubation Performed on Fetus No.	Hydroxylated Metabolites Isolated (in the Case of Sulfoconjugated Metabolites, the Sulfate Group is not indicated)	Specific Enzyme Activity (nmol/mg of protein per min)	t_R (SE-30)	Molecular Ion	Base Peak	Other Prominent Peaks	Ref
Dehydroepiandrosterone	1,3,6,9,[b] 21,22	7α-Hydroxydehydroepiandrosterone	0.128	0.59	448	358		Björkhem *et al.*, 1970
		7β-Hydroxydehydroepiandrosterone	0.060	0.72	448	358		Björkhem *et al.*, 1970
		16α-Hydroxydehydroepiandrosterone	0.966	0.82	448	214	129,196, 199,304	Gustafsson *et al.*, 1969
		X-Hydroxydehydroepiandrosterone	0.019	0.72	448	129	143,169, 229,268, 319,358	Gustafsson *et al.*, 1969
		Total amount of 16-oxygenated dehydroepiandrosterone	3.56					
Dehydroepiandrosterone sulfate	1,3,6,9,[b] 21,22	16α-Hydroxydehydroepiandrosterone	1.26	0.82	448	214	129,196, 199,304	Gustafsson *et al.*, 1969
		Total amount of 16-oxygenated dehydroepiandrosterone	4.20					
Estrone	13,15,19[b]	15α-Hydroxyestrone	0.016	1.00	430	430	129,286, 312,402	
		16α-Hydroxyestrone	0.038	0.91	430	286	415,430	
Estrone sulfate	13,15[b]	15α-Hydroxyestrone	0.022	1.00	430	430	129,286, 312,402	
		16α-Hydroxyestrone	0.384	0.91	430	286	218,244, 286,430	
		16β-Hydroxyestrone	0.350	0.97	430	286	129,415, 430	
		18-Hydroxyestrone	0.025	1.23	430	400	218,312, 340,342	
Estradiol	4,8,15,17, 21[b]	None	< 0.001					
Estradiol 17-sulfate	4,8,14,15[b]	None	< 0.001					
Estradiol 3-sulfate	20	15α-Hydroxyestradiol	0.124[c]	1.09	504	504	191,217, 245	
		16α-Hydroxyestradiol	0.048[c]	1.23	504	129	296,311, 386,504	Einarsson *et al.*, 1973b
Pregnenolone	4,6,10,17[b]	16α-Hydroxypregnenolone	0.741	1.21	476	129	157,159, 172,296	Gustafsson *et al.*, 1968a
Pregnenolone sulfate	4,6,10,17[b]	15β-Hydroxypregnenolone	0.142	1.15	476	281	129,157, 253,296	
		16α-Hydroxypregnenolone	1.19	1.26	476	129	157,159, 172,296	Gustafsson *et al.*, 1968a
Deoxycorticosterone	18,20[b]	None	< 0.001					
Deoxycorticosterone sulfate	18,20[b]	None	< 0.001					
Cholesterol	12,15[b]	None	< 0.001					
Cholesteryl sulfate	12,15[b]	None	< 0.001					

[a] References are given to publications where the identifications are described in detail. [b] When the substrate was incubated with microsomal preparations from different fetuses, the specific enzyme activity was calculated as the mean value obtained from the different incubations. [c] This incubation was performed under nonenzymatic conditions; the specific enzyme activities given therefore represent minimum values.

liver microsomal fractions from different human fetuses are summarized in Table IV. The lower detection limit of the method was 0.001 nmol of product formed/mg of microsomal protein per min. The only identifications discussed in detail below are those of hydroxylated metabolites not previously described. References are given to publications where the identifications of the other hydroxylated products are found.

Incubations of Free and Sulfoconjugated Testosterone. Free testosterone was converted to 1β-, 2α-, 2β-, 6β- (main metabolite), and 18-hydroxytestosterone. In contrast to this, testosterone sulfate was mainly metabolized to 2β-hydroxytestosterone sulfate; small amounts of 2α-hydroxytestosterone sulfate were also found.

Incubations of Free and Sulfoconjugated 5α-Dihydrotestosterone. The only hydroxylated metabolite formed from free 5α-dihydrotestosterone was 18-hydroxy-5α-dihydrotestosterone. One single hydroxylated metabolite was also formed when 5α-dihydrotestosterone sulfate was incubated with human fetal liver microsomes. The silyl ether of this compound has a molecular ion (M) at *m/e* 450, a base peak at *m/e* 435 (M − 15), and a prominent peak at *m/e* 129. The relative retention time (t_R) on SE-30 was 0.95. A very similar mass spectrum was yielded by the silyl ether of the major product (t_R = 0.96) formed upon hydrogenation of 4-hydroxytestosterone, using palladium on charcoal as catalyst (Gustafsson and Sjövall, 1968a). Based on these considerations the hydroxylated metabolite formed was identified as 4-hydroxy-5α-dihydrotestosterone sulfate.

Incubations of Free and Sulfoconjugated 5α-Androstane-3,17β-diol. The major products formed from 5α-androstane-3α,17β-diol were the 6α-, 16α-, and 18-hydroxylated derivatives. Two other hydroxylated metabolites were formed in smaller amounts. One of these gave a mass spectrum indicating an androstane-3,15,17-triol configuration. When the silyl ether of this metabolite was analyzed by gas–liquid chromatography using 3% EGSP-Z as stationary phase, it had a t_R of 0.39, characteristic of the isomer 5α-androstane-3α,15α,17β-triol (Gustafsson and Ingelman-Sundberg, 1974). The other hydroxylated metabolite formed from 5α-androstane-3α,17β-diol was also an androstanetriol as evident from the molecular ion of its silyl ether (at *m/e* 524). The base peak was at *m/e* 215 and prominent peaks were present at *m/e* 434 (M − 90), 395 (M − 129), 393 (M − 131), and 255 (M − (2 × 90 + 89)). The t_R on SE-30 was 0.87. Hydrogenation of 4-hydroxytestosterone with palladium on charcoal as catalyst, followed by reduction with $NaBH_4$, resulted in the formation of several isomers of androstane-3,4,17-triol the silyl ethers of which had t_R's of 0.76, 0.87, and 1.15. The silyl ethers of these derivatives yielded mass spectra very similar to that of the silyl ether of the isolated hydroxylation product. This compound was therefore identified as 5α-androstane-3,4,17β-triol.

When the 17-sulfate ester of 5α-androstane-3β,17β-diol was incubated with microsomes from human fetal liver, the major metabolite formed was 5α-androstane-2α,3β,17β-triol. Hydroxylation at position 4 also occurred and was of the same order of magnitude as when free 5α-androstane-3α,17β-diol was used as substrate.

Disulfurylated 5α-androstane-3α,17β-diol could not serve as substrate for the microsomal hydroxylase systems present in human fetal liver.

Incubations of Free and Sulfoconjugated Androsterone. 16α- and 18-Hydroxylated androsterones were formed in high yields when free androsterone was incubated. Minor amounts of 6-hydroxyandrosterone were also formed. In contrast to this, the only hydroxylase system active on androsterone sulfate was the 18-hydroxylase system, which was about twice as active on the sulfurylated substrate as on the free substrate.

Incubations of Free and Sulfoconjugated Dehydroepiandrosterone. The predominant hydroxylated metabolite formed from free dehydroepiandrosterone was 16α-hydroxydehydroepiandrosterone. Smaller amounts of 7α- and 7β-hydroxydehydroepiandrosterone and an unknown hydroxylated metabolite were also found. In addition to these hydroxylated products, substantial quantities of 3β,17β-dihydroxy-5-androsten-16-one (Gustafsson *et al.*, 1969) were also isolated from the incubation mixtures. This steroid was probably formed from 16α-hydroxydehydroepiandrosterone (Gustafsson *et al.*, 1969) and Table IV therefore also gives the total amount of 16-oxygenated products (16α-hydroxydehydroepiandrosterone + 3β,17β-dihydroxy-5-androsten-16-one) formed from free dehydroepiandrosterone.

The sulfate ester of dehydroepiandrosterone was only hydroxylated in position 16α but also this substrate was metabolized into relatively large amounts of (sulfurylated) 3β,17β-dihydroxy-5-androsten-16-one. When the total amounts of 16-oxygenated products formed from free and sulfoconjugated dehydroepiandrosterone were compared, dehydroepiandrosterone sulfate was found to be a somewhat more efficient precursor than the free steroid.

Incubations of Free and Sulfoconjugated Estrone. Free estrone was metabolized into 15α- and 16α-hydroxyestrone. The 16α-hydroxylase system was about ten times more efficient with estrone sulfate as substrate. It cannot be excluded that the 16β-hydroxyestrone sulfate also formed from this substrate originated from 16α-hydroxyestrone sulfate and was formed by oxidation and reduction at C-16 (Gustafsson *et al.*, 1969). In addition to the two major 16-hydroxylated products, estrone sulfate was also metabolized into two minor products. One of these was identified as 15α-hydroxyestrone sulfate. The silyl ether of the other metabolite yielded a mass spectrum with a molecular ion at *m/e* 430 (indicating a hydroxyestrone configuration), a base peak at *m/e* 400 (M − 30), and prominent peaks at *m/e* 228, 312, 340 (M − 90), and 342 (M − 88). Loss of 30 mass units from the molecular ion is typical of 18-trimethylsiloxy-17-oxosteroids and 18-trimethylsiloxy-3-oxo-Δ^4-steroids (Gustafsson and Lisboa, 1970c). Since the latter configuration is not possible in the present case, the metabolite formed from estrone sulfate was identified as 18-hydroxyestrone sulfate.

Incubations of Free and Sulfoconjugated Estradiol. Despite several attempts it was not possible to demonstrate any hydroxylation of estradiol by liver microsomal preparations from human fetuses. Neither could estradiol 17-sulfate serve as substrate for the hydroxylase systems in question. On the other hand, the 3-sulfate ester of estradiol turned out to be a good substrate for these enzyme systems and was hydroxylated in positions 15α (predominant reaction) and 16α.

Incubations of Free and Sulfoconjugated Pregnenolone. The only hydroxylated metabolite formed from pregnenolone was 16α-hydroxypregnenolone. The 16α-hydroxylase system was also active on sulfoconjugated pregnenolone. As was observed with free and sulfoconjugated dehydroepiandrosterone, the sulfate ester was a somewhat better substrate for the 16α-hydroxylase system than the free steroid.

Table V: K_m Values for the 7α- and 7β-Hydroxylase Systems Active on Free Dehydroepiandrosterone and for the 16α-Hydroxylase Systems Active on Free and Sulfoconjugated Dehydroepiandrosterone and Pregnenolone.

Substrate	Fetus No.	K_m(μM) 16α	7α	7β
Dehydroepiandrosterone	21 + 22	26.3	40.0	38.4
Dehydroepiandrosterone sulfate	21 + 22	125		
Pregnenolone	16	244		
Pregnenolone sulfate	16	238		

In addition to the 16α-hydroxylated metabolite, pregnenolone sulfate was metabolized into a second hydroxylated derivative. The t_R of the silyl ether of this compound on SE-30 was 1.19. Its mass spectrum showed a molecular ion at *m/e* 476, a base peak at *m/e* 281 (M − (90 + 15)), and prominent peaks at *m/e* 296 (M − (2 × 90)), 253, 157, and 129. Peaks at *m/e* 157 and 129 are found in mass spectra of 15-trimethylsiloxy-C_{21} steroids with an acetyl side chain and probably represent fragmentations in ring D and the side chain (Gustafsson and Sjövall, 1968a; Eriksson and Gustafsson, 1970). In order to obtain a comparable reference steroid, 15-oxopregnenolone was reduced by $NaBH_4$ and subsequently oxidized by 20β-hydroxysteroid oxidoreductase from *Streptomyces hydrogenans* as described previously (Lisboa and Gustafsson, 1968b). Two products with a hydroxypregnenolone structure were found in the reaction mixture. The silyl ethers of both of these compounds yielded mass spectra that were very similar to that of the silyl ether of the isolated hydroxylation products of pregnenolone sulfate. The t_R's of the silyl ethers of the prepared reference compounds were 1.34 and 1.19 (major compound). The ratio between these t_R's (1.13) is close to the ratio between the t_R's of 3β,15α- and 3β,15β-dihydroxy-5α-pregnan-20-one silyl ethers (1.10; *cf.* Eriksson and Gustafsson, 1970). Based on these considerations, the isolated hydroxylation product of pregnenolone sulfate was identified as 15β-hydroxypregnenolone sulfate.

Incubations of Free and Sulfoconjugated Deoxycorticosterone and Cholesterol. Neither free nor sulfoconjugated deoxycorticosterone was hydroxylated by liver microsomal preparations from human fetuses. The same was true for cholesterol and cholesteryl sulfate.

Kinetic Aspects of Human Fetal Liver Hydroxylation of Free and Sulfoconjugated Dehydroepiandrosterone and Pregnenolone. Table V summarizes the K_m values determined for the hydroxylase systems active on free and sulfoconjugated dehydroepiandrosterone and pregnenolone. The K_m value for the 16α-hydroxylase system was almost identical with pregnenolone and pregnenolone sulfate as substrates (about 240 μM). However, the V_{max} for the 16α-hydroxylase system was about 1.6 times higher with the sulfate ester as substrate than with the free steroid. The V_{max} for the 16α-hydroxylase system active on dehydroepiandrosterone was also somewhat higher (about 1.3 times) with the sulfate ester as substrate than with the free steroid. However, free dehydroepiandrosterone (K_m = 26 μM) has a much higher affinity to the 16α-hydroxylase system than dehydroepiandrosterone sulfate (K_m = 125 μM). K_m values for the 7α- and 7β-hydroxylase systems active on free dehydroepiandrosterone were similar to that of the 16α-hydroxylase system active on the same substrate (about 40 μM).

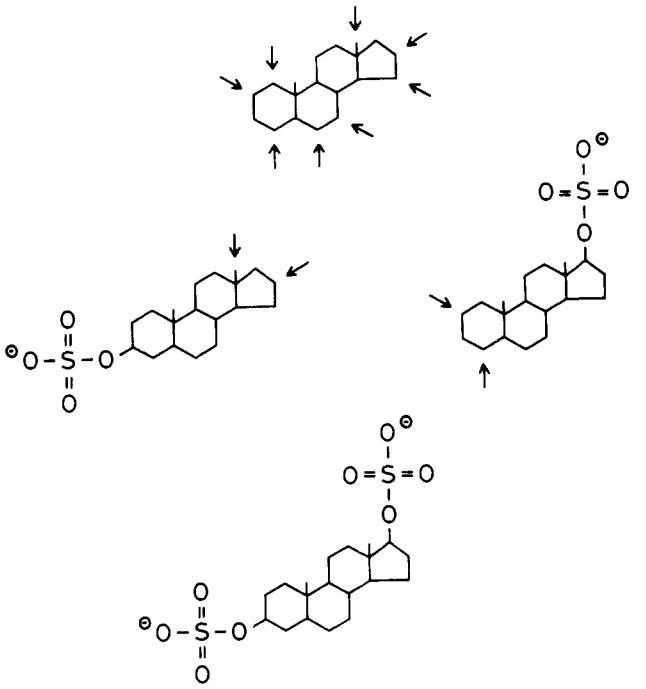

FIGURE 1: Schematic representation of positions (indicated by arrows) in the steroid molecule hydroxylated by the human fetal liver microsomal hydroxylase system. For explanations, see text.

In a separate experiment dehydroepiandrosterone was incubated with fetal liver microsomes in the presence of two different concentrations of dehydroepiandrosterone sulfate. Lineweaver–Burk plots showed that all hydroxylase systems active on free dehydroepiandrosterone were competitively inhibited by dehydroepiandrosterone sulfate. The inhibition constants varied from 185 to 270 μM (7α-hydroxylase, 229 μM; 7β-hydroxylase, 270 μM; and 16α-hydroxylase, 185μM).

Discussion

The present investigation has shown that steroid sulfates are hydroxylated in a number of positions by the microsomal fraction from human fetal liver. However, important differences were noted in the mode of metabolism of free compared to sulfurylated steroids. Free steroids were hydroxylated in positions all around the steroid molecule, *i.e.*, in positions 1β, 2α, 2β, 4, 6α, 6β, 7α, 7β, 15α, 16α, and 18. Steroid sulfates, on the other hand, were only hydroxylated in positions 2α, 2β, and 4 (in the case of a steroid 17β-monosulfate) or in positions 15α, 15β, 16α, and 18 (in the case of a steroid 3-monosulfate). Thus hydroxylation of steroid sulfates only occurred at a pronounced distance from the sulfate group (Figure 1). In accordance with this interpretation, a 3,17-disulfurylated C_{19} steroid substrate was not found to be metabolized by the microsomal fraction. Figure 2A suggests schematically how the hydrophobic nature of human fetal liver microsomal cytochrome P-450 only allows interaction with the less hydrophilic nonsulfurylated end of the steroid sulfate. The occurrence of two hydrophilic ends in the substrate makes the formation of an enzyme–substrate complex impossible.

All hydroxylase activities active on free dehydroepiandrosterone, *i.e.*, the 7α-, 7β, and 16α-hydroxylase activities, were competitively inhibited by the presence of dehydroepi-

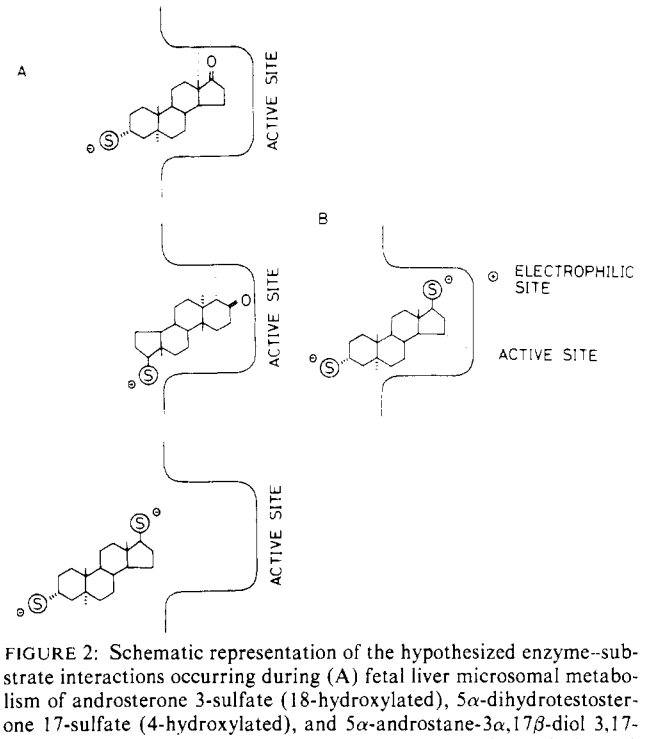

FIGURE 2: Schematic representation of the hypothesized enzyme–substrate interactions occurring during (A) fetal liver microsomal metabolism of androsterone 3-sulfate (18-hydroxylated), 5α-dihydrotestosterone 17-sulfate (4-hydroxylated), and 5α-androstane-3α,17β-diol 3,17-disulfate (not hydroxylated) (for explanations, see text) and during (B) hydroxylation of 5α-androstane-3α,17β-diol 17-sulfate in position 15β by female rat liver microsomal cytochrome P-450. In the latter case an ionic interaction between the negatively charged sulfate group and an electrophilic site on the enzyme is proposed.

androsterone sulfate. This indicates that both the sulfate ester and the free steroid are metabolized by the same enzyme system. In the case of pregnenolone, the free and the sulfurylated substrates were equally well bound to the active site of the hydroxylase system, as judged by the almost identical K_m values for these substrates with respect to 16α-hydroxylation. Free dehydroepiandrosterone, on the other hand, seemed to have a greater affinity to the 16α-hydroxylating enzyme system than dehydroepiandrosterone sulfate.

The simple type of enzyme–substrate interaction outlined above, however, is not sufficient to explain all the qualitative and quantitative differences between the metabolism of free as compared to sulfurylated steroid substrates. Free androsterone was mainly 16α-hydroxylated but also 6- and 18-hydroxylated whereas androsterone sulfate was only 18-hydroxylated. Free pregnenolone was only hydroxylated in position 16α whereas the sulfate ester was also hydroxylated in position 15β. Similarly, free estrone was hydroxylated in positions 15α and 16α whereas estrone sulfate was both 15α-, 16α-, and 18-hydroxylated. Finally, the ring A hydroxylation of free testosterone occurred in positions 1β, 2α, and 2β (ratio of 2β-/2α-hydroxytestosterone = 1) whereas testosterone sulfate was only hydroxylated in positions 2α and 2β (ratio of 2β-/2α-hydroxytestosterone = 62). These differences between free and sulfurylated steroids may be due to a directing influence of the negatively charged sulfate group on the position of the steroid substrate on the enzyme surface. In this way slightly different parts of the same region of the steroid substrate are exposed to the active site of the hydroxylating enzyme system. These findings are in further support of the presence of a highly unspecific, steroid hydroxylating species of cytochrome P-450 in human fetal liver microsomes.

An interesting consequence of the directing influence of the sulfate group is seen in the case of estradiol and estradiol 3-sulfate. Whereas the free steroid could not serve as substrate of the microsomal hydroxylase systems, the sulfate ester was both 15α- and 16α-hydroxylated. Together with the results obtained with free and sulfoconjugated estrone (see above) these findings indicate that the main metabolic pathway to 16α-hydroxylated estrogens, such as estriol, in the human fetal liver, proceeds *via* 3-sulfurylated compounds.

A totally different species of cytochrome P-450 seems to be involved in the hydroxylation of steroid sulfates in liver microsomes from female rats (Gustafsson and Ingelman-Sundberg, 1975). In this case hydroxylations (usually in position 15β, sometimes also in position 7β) take place in the immediate vicinity of the 17β- and 21-sulfate groups of C_{19} and C_{21} steroids, respectively. Furthermore, a highly polar substrate such as 5α-androstane-3α,17β-diol 3,17-disulfate has a higher affinity for the 15β-hydroxylating enzyme system than 5α-androstane-3α;17β-diol 17-sulfate.

Lu *et al.* (1973) have described the separation of the microsomal hydroxylation system in rat liver metabolizing drugs and steroids into fractions containing cytochrome P-450, NADPH–cytochrome P-450 reductase, and phospholipid. The specificity for hydroxylation reactions has been found to reside in the cytochrome fraction. It therefore seems adequate to interpret the results described above with female rats as indicating the presence of a specific hydrophilic steroid sulfate-hydroxylating species of cytochrome P-450 in liver microsomes from female rats (Figure 2B). The specificity of this hydroxylase system is further indicated by its unique type of regulation when compared to other hydroxylase systems in rat liver (Gustafsson and Ingelman-Sundberg, 1974).

The occurrence in human fetal liver of a multipotent, unspecific, hydrophobic "bulk" of cytochrome P-450 capable of hydroxylating steroid sulfates and possibly also sulfurylated drugs is probably related to the large amounts of sulfoconjugated steroids in the fetus (Huhtaniemi *et al.* 1970). The high sulfurylase activity of human fetal tissues may be considered to be a mechanism of detoxification of drugs and hormones. In contrast to the adult, however, the fetus cannot excrete these compounds in a normal way; excretion occurs into the amniotic fluid and "excretions" of the fetus can recirculate by ingestion. Furthermore, the sulfates are reversibly formed and may be split in the feto-placental unit to release the active product again. This potential danger of accumulation of metabolites is efficiently counteracted by the presence of a sulfate-hydroxylating enzyme system in the fetal liver that completes the detoxification process in an irreversible manner.

Acknowledgments

We are grateful to Dr. S. Fiano and Dr. L. Nilsson for valuable aid during the performance of this study.

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Glucocorticoid Receptors in Mouse Mammary Tumors: Specific Binding to Nuclear Components†

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ABSTRACT: The specific interaction of glucocorticoids with nuclei of mouse mammary tumor was studied *in vitro* by incubation of the tissue with [^{3}H]dexamethasone at 25°. It was demonstrated that the mammary tumors contain a limited number of specific nuclear binding sites which were saturated with low hormone concentrations (10^{-8} M). The concentrations of specific binding sites in the nuclei were related to the concentration of cytoplasmic binding sites of unincubated tissues and varied between individual tumors. The binding component in the nuclei appeared to be a protein and was easily solubilized with 0.4 M KCl containing buffers. The ability of various corticoids to block the nuclear localization of the steroid correlated well with their glucocorticoid potency. Estradiol and progesterone at concentrations of 10^{-6} M were also effective in competing for the glucocorticoid receptor binding sites. However, while the glucocorticoids such as hydrocortisone and corticosterone translocated to nuclear sites also specific for dexamethasone, estradiol and progesterone competed for the cytoplasmic binding sites and did not translocate to the nucleus. The possible significance of the interaction of various steroids with the glucocorticoid receptors in mammary tumors is discussed.

It is currently believed that an initial step in the mechanism of steroid hormone action involves the specific binding of the hormones to the receptor proteins in target cells (Raspe, 1971). In the case of glucocorticoid hormones, such receptors have been demonstrated in liver (Beato *et al.*, 1971), thymus (Munck and Wira, 1971), kidney (Funder *et al.*, 1973), fetal lung (Giannopoulous, 1973a,b; Ballard and Ballard, 1972; Toft and Chytil, 1973), hepatoma cells (Baxter and Tomkins, 1971), steroid sensitive lymphoma cells (Baxter *et al.*, 1971), normal mammary glands (Shyamala, 1973), and pituitary tumor cells (Watanabe *et al.*, 1973, 1974).

Corticosteroid hormones are directly involved in milk secretion in mice (Lyons *et al.*, 1958) and it has been suggested that the hormonal influence, while not inductive, is a permissive one which is essential for mammary tumor ap-

† From the Department of Zoology, the Cancer Research Laboratory, University of California, Berkeley, California 94720. *Received May 13, 1974.* This investigation was supported by Public Health Service Grant CA-05388 and by Cancer Research funds of the University of California.