Chemical and Biochemical Studies on 18-Hydroxyoestrone

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1. 18-Hydroxyoestrone was reduced by NaBH_4 in methanol, giving 18-hydroxyoestradiol-17 α and 18-hydroxyoestradiol-17 β in the ratio 3:7. 2. Treatment of 18-hydroxyoestrone with a strong alkali yielded 18-noroestrone; however, the 18-hydroxyoestradiols did not undergo transformation to their respective 18-nor derivatives. 3. All the 18-hydroxylated oestrogens were stable under acid conditions. They formed Kober chromogens: the chromogenicity of 18-hydroxyoestrone was only one-third that of the 18-hydroxyoestradiols and oestriol. 4. Paper-, thin-layer- and gas-liquid-chromatographic systems for the characterization of these compounds are described. 5. An examination of the mass spectra revealed peaks characteristic of the substituted carbon atoms. Definite assignment of the 17 α - and 17 β -hydroxyl groups of the epimeric 18-hydroxyoestrogens was possible by characteristic fragmentation of the free steroids. Further, the configuration of 18-hydroxyoestradiol-17 β was confirmed by the formation of the dimethylsilyldioxy derivative of the 3-methylether of the steroid. 6. Both rat and rabbit liver slices reduced 18-hydroxyoestrone to 18-hydroxyoestradiol-17 β and some other labile, polar metabolites with properties similar to 2-hydroxylated oestrogens. No formation of 18-hydroxyoestradiol-17 α *in vitro* was observed. 7. The results are discussed with respect to the possible influence of the 18-hydroxyl group on reactions at C-17, as well as the reactions of 18-hydroxylated oestrogens with strong acid (Kober reactions) and alkali.

Endogenous 18-hydroxyoestrone was first characterized after its isolation from extracts of human pregnancy urine (Loke *et al.*, 1959). Loke *et al.* (1957) had reported that ox adrenal homogenates metabolized oestrone to a compound with properties similar to those of 18-hydroxyoestrone, but subsequent attempts to repeat this observation failed (Loke, 1958; Sneddon & Marrian, 1963). After the incubation of human adrenal tissue with labelled oestrone, Knuppen *et al.* (1965) found a radioactive metabolite with chemical and chromatographic properties similar to the 18-hydroxyoestrone described by Loke *et al.* (1959). These and further studies were hampered by the lack of authentic standards until Baldwin *et al.* (1968) described a photolytic method for the synthesis of 18-hydroxyoestrone. The present paper is concerned with the description of some chemical properties of authentic 18-hydroxyoestrone and its derivatives, as well as its metabolism by slices of liver from rat and rabbit.

Methods

Steroids

18-Hydroxyoestrone [3,18-dihydroxy-1,3,5(10)-oestratrien-17-one], obtained through the courtesy of Dr. G. F. Marrian, was prepared by the method

of Baldwin *et al.* (1968) and recrystallized from ethanol before use. Oestrone and oestradiol-17 β were purchased from Schering A.G., Berlin, Germany, and were also recrystallized. All other steroids were standard commercial preparations: 16-epioestriol [1,3,5(10)-oestratriene-3,16 β ,17 β -triol], 17-epioestriol [1,3,5(10)-oestratriene-3,16 α ,17 α -triol], and 2-methoxyestriol [2-methoxy-1,3,5(10)-oestratriene-3,16 α ,17 β -triol].

Paper chromatography

Paper strips (Schleicher and Schüll 2043b paper; 45cm $\times$ 15cm) were washed with methanol for 24h before immersion in methanol-formamide (1:1, v/v). They were then blotted with filter paper and dried in a horizontal position at room temperature for 15 min. All chromatograms were equilibrated for at least 30 min at $25 \pm 2^\circ\text{C}$ and then developed with one of the following solvent systems: P₁, monochlorobenzene-ethyl acetate (3:1, v/v); P₂, monochlorobenzene-ethyl acetate (1:3, v/v); or P₃, chloroform-ethyl acetate (5:1, v/v). After development, they were dried in a draught oven at 70°C for 1–2h to remove the formamide. The appropriate areas were then cut out from the paper and thoroughly extracted with methanol at room temperature. The positions of the reference oestrogens were located on the paper strips by Folin-Ciocalteu reagent (Mitchell & Davies, 1954).

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Thin-layer chromatography

Commercially prepared (E. Merck A.G., Darmstadt, Germany) 20cm×20cm plates, coated with silica gel G (layer thickness 0.25mm) were used after being washed overnight with chloroform-methanol (9:1, v/v). The chromatograms were developed at room temperature with one of the following solvent systems: T₁, ethyl acetate-cyclohexane-ethanol (45:45:10, by vol.); T₂, chloroform-methanol (9:1, v/v); or T₃, chloroform-acetone (9:1, v/v). The plates were dried in air; the areas to be eluted were scraped into glass funnels containing a fine sinter and were eluted three times with 5ml of chloroform-methanol (9:1, v/v). Reference steroids were located by conc. H₂SO₄ or by a 40% (v/v) H₂SO₄ spray solution, after heating for 10min at 100°C.

Gas-liquid chromatography

The oestrogens were chromatographed as trimethylsilyl ether derivatives, under the following conditions: column 180cm×0.3cm; 3% OV-1 or 3% OV-17 on Chromosorb WHP (100-120mesh); inlet temperature 250°C; column temperature 220°C; N₂ flow rate 35ml/min. The Techmation Mikrotek MT 220 gas chromatograph was equipped with a double-flame ionization detector. The oestrogens (100-500ng) were injected in 1-5μl portions of the silylating reagent. Retention times were measured relative to 5α-cholestane.

Mass spectrometry

Investigations were carried out with an LKB 9000 Gas-Chromatograph-Mass Spectrometer. The gas-chromatographic conditions were: column, 180cm×0.3cm; 3% OV-1 on Chromosorb WHP (100-120 mesh); column temperature 220°C; temperature of the separator 210°C. The following conditions were set on the mass spectrometer: temperature of the ion source 250°C; ionization energy 20eV (free steroids) and 70eV (trimethylsilyl ether); ionization current 60μA. Free steroids without the protection of the hydroxyl groups were introduced into the ion source by means of a direct inlet system.

Chemical reactions and derivative formations

Regisil [NO-bis-(trimethylsilyl)trifluoroacetamide, containing 1% chlorotrimethylsilane; 50-100μl; Regis Chemical Co., Chicago, Ill., U.S.A.] was added to approx. 10μg of oestrogen and the mixture heated at 65°C for 10min to effect silylation. The 3-methyl-17β,18-dimethylsildioxy derivative of 18-hydroxy-oestradiol-17β was prepared by adding a solution of diazomethane in diethyl ether (1ml) to a solution of the steroid in methanol (1ml). After 1h solvent and

reagent were evaporated and the 3-methyl ether obtained was treated with a mixture of dichlorotrimethylsilane (50μl) and pyridine (50μl) for 1h at room temperature (Kelly, 1969). G.l.c. and g.l.c.-mass spectrometry were carried out by injecting portions of the reaction mixture directly into the gas chromatograph. NaBH₄ reduction in methanol was performed at room temperature as described by Knuppen *et al.* (1967). The 18-hydroxylated oestrogens were exposed to HCl-methanol for 1h (Knuppen *et al.*, 1966), to 3M-NaOH at room temperature for 14h (Knuppen *et al.*, 1965) or to 6M-H₂SO₄ at room temperature for 14h. These reaction mixtures were neutralized with 10% (v/v) acetic acid or a saturated NaHCO₃ solution and their contents were extracted with freshly distilled ether or ethyl acetate. After being washed to neutrality with water, the solvent extracts were dried over anhydrous Na₂SO₄ and then evaporated to dryness *in vacuo*. Reaction conditions for the formation of acetone derivatives were as described by Heikkilä & Adlercreutz (1970), by using chlorotrimethylsilane (Fluka A.G. Chemische Fabrik, Buchs, Switzerland) in freshly distilled acetone. Colour reactions with Haenni's Reagent B (Schott & Katzman, 1964), were measured at 520nm with a Beckman DB spectrophotometer. The Kober reaction was carried out as described by Nocke (1961).

Miscellaneous

U.v. spectra were recorded in ethanol (Uvasol grade, E. Merck A.G.) with a Beckman model DK2-A recording spectrophotometer, with optically matched quartz cuvettes. Quantitative measurements of oestrogens by u.v. spectroscopy were done at 280nm by using a molar extinction coefficient (ϵ) = 2200litre·mol⁻¹·cm⁻¹ for all oestrogens, including the 18-hydroxylated derivatives (Loke *et al.*, 1959). Adult male animals were fed on Altromin diet but were starved the night before use. All reagents and solvents were of analytical grade.

Incubation procedure with liver tissue

The animals were stunned by a sharp blow behind the head and decapitated; their blood was allowed to drain for approx. 2min. The livers were removed and washed in ice-cold 0.25M-sucrose. Slices were prepared by the method of Deutsch (1936). In each experiment, slices (400mg) were incubated in Krebs-Ringer phosphate saline (pH 7.4; 4.0ml) (Krebs & Henseleit, 1932), containing glucose (20mm) and 18-hydroxyoestrone (100μg, previously dissolved in two drops of propylene glycol). Control incubations contained substrate and buffer (reagent blank) or tissue and buffer (tissue blank). Duplicate incubations were carried out in air at 37°C for 1h. The mixtures

were extracted twice with an equal volume of ether-chloroform (3:1, v/v); the combined extracts were washed with water (2ml) and then evaporated to dryness *in vacuo*. The residues, dissolved in methanol (0.1ml), were applied to formamide-impregnated papers for chromatography.

Results

Chemical properties of the 18-hydroxylated oestrogens

Table 1 summarizes the results of various treatments of the 18-hydroxylated oestrogens. Treatment of 18-hydroxyoestrone with NaBH₄ in methanol resulted in formation of both 18-hydroxyoestradiol-17 α [1,3,5(10)-oestratriene-3,17 α ,18-triol] and 18-hydroxyoestradiol-17 β [1,3,5(10)-oestratriene-3,17 β ,18-triol] in the ratio 3:7. The epimers were identified by their chromatographic mobilities (Table 2) and finally by mass spectrometry (Table 3). When 18-hydroxyoestrone was treated with strong alkali, 18-noroestrone and presumably formaldehyde (Loke *et al.*, 1959) were formed; we also observed Folin-Ciocalteu-positive compounds on the chromatograms developed in monochlorobenzene-ethyl acetate (3:1, v/v), whose mobilities (4–6cm/h) were between those of 18-noroestrone (7.0cm/h) and 18-hydroxyoestrone (1.4cm/h). These compounds remained unidentified.

It was noteworthy that the 18-nor derivatives of 18-hydroxyoestradiol-17 α and 18-hydroxyoestradiol-17 β were not formed after treatment with strong alkali. All the 18-hydroxylated oestrogens tested were stable to acid treatment.

An examination of the Dreiding models indicated that the β -orientation of the C-17 hydroxyl group of 18-hydroxyoestradiol-17 β might be sufficiently close to the 18-hydroxyl group to allow the formation of an acetonide derivative, similar to *cis*-glycols such as 16-epioestrinol. However, 18-hydroxyoestradiol-17 β did not form an acetonide derivative. In contrast a 'siliconide' (Kelly, 1969), which is a compound very similar to an acetonide, could be formed. To obtain the cyclic 17 β ,18-dimethylsildioxy derivative, previous protection of the phenolic 3-hydroxyl group of 18-hydroxyoestradiol-17 β by methylation was necessary. The derivative was identified by combined g.l.c.-mass spectrometry. It should be pointed out that, by the formation of the cyclic derivative, it is possible to assign the structure of the compound to the 17 β ,18-hydroxy configuration. As expected from steric considerations, only the 18-hydroxyoestradiol-17 β forms a sildioxy derivative. An attempt to form a similar derivative of 18-hydroxyoestradiol-17 α failed. No 18-hydroxylated oestrogens, in particular 18-hydroxyoestradiol-17 α , gave a positive reaction with

Table 1. *Summary of the chemical properties of 18-hydroxylated oestrogens*

Experimental details of the various treatments and identification procedures are given in the Methods section. Abbreviations: g.l.c., gas-liquid chromatography; t.l.c., thin-layer chromatography; p.c., paper chromatography; m.s., mass spectroscopy.

Oestrogen	Experimental condition	Result	Identification
18-Hydroxy-oestrone	Silylation	Formation of the trimethylsilyl ether	G.l.c. and m.s.
	Methanol-HCl	No change	P.c.
	6 M-H ₂ SO ₄	No change	P.c.
	3 M-NaOH	Formation of 18-noroestrone and formaldehyde as well as other unidentified non-polar compounds	P.c., t.l.c., g.l.c. and m.s.
18-Hydroxy-oestradiol-17 β	NaBH ₄ -methanol	Formation of 18-hydroxyoestradiol-17 α and 18-hydroxyoestradiol-17 β (ratio 3:7)	P.c., t.l.c., g.l.c., m.s. and u.v. spectra
	Kober reaction	Positive; chromogenicity 29% that of oestrinol	—
	Silylation	Formation of the trimethylsilyl ether	G.l.c. and m.s.
	Methylation in position 3 and reaction with dichlorodimethylsilane	3-Methyl-17 β ,18-dimethylsildioxy derivative	G.l.c. and m.s.
18-Hydroxy-oestradiol-17 α	Trimethylchlorosilane-acetone	No formation of an acetonide derivative	P.c.
	3 M-NaOH	No formation of 18-nor compounds	P.c.
	Kober reaction	Positive; chromogenicity equal to that of oestrinol	—
	Silylation	Formation of the trimethylsilyl ether	G.l.c. and m.s.
18-Noroestrone	3 M-NaOH	No formation of 18-nor compounds	P.c.
	Kober reaction	Positive; chromogenicity equal to that of oestrinol	—
	Haenni reaction (Reagent B)	Negative	—
	Silylation	Formation of the trimethylsilyl ether	G.l.c. and m.s.

Table 2. *Chromatographic mobilities of 18-hydroxylated oestrogens*

Retention times, relative to 5 α -cholestane, in g.l.c. were recorded, by using a 1.8 m column of 3% OV-1 at 220°C and a 1.8 m column of 3% OV-17 at 235°C, and the trimethylsilyl ether derivatives of the oestrogens. Paper chromatography (p.c.) was carried out on formamide-impregnated paper with the following systems: P₁, monochlorobenzene-ethyl acetate (3:1, v/v); P₂, monochlorobenzene-ethyl acetate (1:3, v/v); P₃, chloroform-ethyl acetate (5:1, v/v). T.l.c. was carried out on silica gel G with the following systems: T₁, ethyl acetate-cyclohexane-ethanol (45:45:10, by vol.); T₂, chloroform-methanol (9:1, v/v); T₃, chloroform-acetone (9:1, v/v). For further details see the Methods section.

Oestrogen	Relative retention time in g.l.c.		Mobility in p.c. system (cm/h)			Mobility in t.l.c. system (R_F values)		
	OV-1	OV-17	P ₁	P ₂	P ₃	T ₁	T ₂	T ₃
Oestrone	0.53	0.98	6.5	—	—	0.63	0.50	0.47
Oestradiol-17 β	0.67	0.72	5.3	—	—	0.53	—	0.30
Oestradiol-17 α	0.60	0.63	—	—	—	—	—	—
Oestriol	—	—	0.5	1.4	1.2	0.25	—	—
16-Epioestriol	—	—	—	—	—	0.39	—	—
17-Epioestriol	—	—	2.6	5.0	4.3	0.42	—	—
2-Methoxyoestriol	—	—	0.9	3.4	1.8	0.25	—	—
18-Hydroxyoestrone	0.88	1.26	1.4	3.8	2.3	0.40	0.24	0.13
18-Noroestrone	0.54	—	7.0	—	—	—	—	—
18-Hydroxyoestradiol-17 β	0.83	0.72	3.1	4.8	3.6	0.40	0.36	0.07
18-Hydroxyoestradiol-17 α	0.90	0.77	0.8	1.5	1.3	—	0.24	—
17 β ,18-Dimethylsildioxy-18-hydroxyoestradiol-17 β 3-methyl ether	0.83	1.52	—	—	—	—	—	—

Haenni's Reagent B. They were all Kober-positive when tested under the condition for oestriol. However, 18-hydroxyoestrone had only 29% of the colour intensity of an equivalent weight of oestriol, whereas the two epimers had the same intensity as oestriol per unit weight.

Chromatographic properties of the 18-hydroxylated oestrogens and their colour reactions on paper and thin-layer chromatograms

A better separation of the 18-hydroxylated oestrogens was obtained in the paper than in the thin-layer systems; in particular, system P₁ was effective in separating the 18-hydroxylated oestrogens from each other as well as from the other oestrogens tested (Table 2). 18-Hydroxyoestradiol-17 β was less polar than 18-hydroxyoestrone and 18-hydroxyoestradiol-17 α in these systems. This was thought to be due to a hydrogen bridge between the 18-hydroxyl and 17 β -hydroxyl groups of this compound.

All the 18-hydroxylated oestrogens gave positive blue colours with Folin-Ciocalteu reagent on paper, but only after exposure to NH₃. H₂SO₄ (40% spray reagent or conc.) produced a yellow-orange colour when used on silica gel G with these compounds. An attempt to differentiate between 18-hydroxyoestradiol-17 α and 18-hydroxyoestradiol-17 β by their colour reactions with phosphomolybdic acid [10% (w/v) in ethanol after heating at 110°C for 20 min on silica gel] (J. K. Findlay, unpublished work) was

not successful; under these conditions, oestradiol-17 α gives a purple colour after 5 min, whereas oestradiol-17 β gives a blue colour only after 15–20 min heating.

G.l.c. properties of trimethylsilyl ether derivatives of the 18-hydroxylated oestrogens

All the 18-hydroxylated oestrogens formed trimethylsilyl ether derivatives of both the primary and secondary alcohols and also of the phenolic hydroxyl groups, and portions of 100–500 ng were separable on a 3% OV-1 column at 220°C or a 3% OV-17 column at 235°C (Table 2). It is noteworthy that 18-hydroxyoestradiol-17 β trimethylsilyl ether had a shorter retention time than its 17 α -epimer; this is further evidence for some interaction between the 17 β -hydroxyl and 18-hydroxyl groups.

Mass-spectrometric properties of the 18-hydroxylated oestrogens

The compounds were examined as the free steroids and as the trimethylsilyl ether derivatives; details are given in Table 3. The mass spectrum of 18-hydroxyoestrone (Fig. 1) shows an $M^+ - 30$ fragment ion of very high relative intensity, which may be formed by a McLafferty rearrangement (Scheme 1). By migration of the 18-hydroxyl hydrogen to the 17-carbonyl group, a cyclic mechanism is initiated which leads to the expulsion of a CH₂=O molecule.

Table 3. *m/e* values and relative intensities of mass spectra of the free 18-hydroxy steroids and their derivatives
For details see the text.

18-Hydroxy-oestrone	18-Hydroxy-oestradiol-17 α	18-Hydroxy-oestradiol-17 β	18-Hydroxyoestrone trimethylsilyl ether	18-Hydroxyoestradiol-17 α trimethylsilyl ether	18-Hydroxyoestradiol-17 β trimethylsilyl ether	17 β ,18-Dimethylsilyldioxy-18-hydroxyoestradiol-17 β 3-methyl ether
<i>m/e</i>	<i>m/e</i>	<i>m/e</i>	<i>m/e</i>	<i>m/e</i>	<i>m/e</i>	<i>m/e</i>
146	78	133	73	103	103	73
160	133	146	103	129	129	75
172	146	160	221	217	217	91
185	160	172	230	232	232	115
213	172	213	285	283	283	147
228	211	226	310	324	324	160
256	239	240	400	414	414	173
286	288	288	430	504	504	358
Relative intensity	Relative intensity	Relative intensity	Relative intensity	Relative intensity	Relative intensity	Relative intensity
10	15	17	35	12	21	66
12	11	8	72	87	84	100
9	11	66	32	52	39	29
17	24	16	62	72	49	27
8	8	20	39	100	28	37
9	14	19	35	100	100	29
100	17	15	20	38	24	19
14	100	100	100	91	84	26

The high intensity of the resulting $M-30$ ion may be explained by the fact that low energy is needed for this process in comparison with other fragmentation reactions. Apart from this characteristic behaviour of 18-hydroxy-17-oxosteroids, some more fragments (m/e 146, 160, 172, 185, 213) are observed which are the same as in the mass spectrum of oestrone (Budzikiewicz *et al.*, 1964).

In contrast with 18-hydroxyoestrone, the mass spectra of 18-hydroxyoestradiol-17 β (Fig. 2) and 18-hydroxyoestradiol-17 α (Fig. 3) show molecular ions of high relative intensities, which form the base peaks of the spectra. In addition to the molecular ions, and a number of peaks (m/e 133, 146, 160, 172, 213) which are typical of the oestrogen molecule, the mass spectra of 18-hydroxyoestradiol-17 α and 18-hydroxyoestradiol-17 β reveal some characteristic features; these make it possible to assign the structure to the α and β isomers. 18-Hydroxyoestradiol-17 α shows fragment ions, resulting from the loss of a molecule of H_2O at C-17 (m/e 270, M^+-18), from a cleavage at C-18 (m/e 257, M^+-31) or from the probable elimination of both the H_2O at C-17 and the $18-CH_2-OH$ (m/e 239, M^+-49) (Scheme 2). On the other hand, 18-hydroxyoestradiol-17 β shows a fragment ion at m/e 240. The formation of this M^+-48 fragment may be explained by a cyclic mechanism, similar to the McLafferty rearrangement (Scheme 3). We conclude from stereochemical considerations that only the 17 β isomer reacts in this way.

The trimethylsilyl derivatives of the 18-hydroxy-oestrogens exhibit mass spectra predominantly characterized by the behaviour of the trimethylsilyl groups, e.g. peaks at M^+-90 and m/e 73. Other fragments, characteristic of the primary silylated alcohol group at C-18, are observed. These include peaks at M^+-103 and m/e 103 as well as $M^+-(90+103)$, which are due to cleavage between C-18 and C-13 (Gustafsson & Sjövall, 1968; Lisboa & Gustafsson, 1969). 18-Hydroxyoestrone trimethylsilyl ether had a characteristic M^+-30 fragment (m/e 400) which seems to be typical for 18-hydroxy-17-oxo steroids (Gustafsson & Lisboa, 1970). This fragment may be formed by the same cyclic mechanism as proposed for the M^+-30 fragment within the mass spectrum of the free steroid. In this case, the reaction starts with a migration of the trimethylsilyl group instead of the hydrogen atom from the 18-oxygen function to the 17-carbonyl oxygen, followed by the expulsion of the CH_2O molecule. It may be seen from a metastable ion at m/e 372.4 (calculated 372.1) that the transition from the molecular ion (m/e 430) to the M^+-30 fragment (m/e 400) is a reaction in one step and cannot be due to a consecutive loss of two methyl radicals from the trimethylsilyl groups. In contrast with the free steroids the mass spectra of the silylated 18-hydroxyoestradiol epimers did not show any significant difference. In addition to the

Fig. 1. Mass spectrum of 18-hydroxyestrone

For details see the text.

Scheme 1.

peaks characteristic of 18-hydroxy steroids, at $M^+ - 103$ (m/e 401) and m/e 103, some fragment ions typical of 17-hydroxyoestrogens (m/e 129 and m/e 373, $M^+ - 131$) can be identified; these are generated by expulsion of ring-D fragments. The mass spectrum of the 3-methyl-17 β ,18-dimethylsildioxy derivative of 18-hydroxyoestradiol-17 β shows a correct molecular weight at m/e 358. Further, there are some peaks at high intensity at m/e 73, 75 and 77, which are possibly formed by cleavage of the cyclic sildioxy structure.

Incubation of 18-hydroxyestrone with liver slices

After incubation of 18-hydroxyestrone with rat liver slices, four phenolic substances were detected, two of which were identified as 18-hydroxyestrone and 18-hydroxyoestradiol-17 β by chromatography in paper, thin-layer and gas-liquid systems as well as by mass spectrometry. The other two substances remained on or near the origin after development in system P₁. The blue colour of these compounds appeared immediately; after the paper was dipped through Folin-Ciocalteu reagent, the unidentified

compounds were labile when eluted from the paper with methanol. Approx. 15% of the substrate was transformed to 18-hydroxyoestradiol-17 β and 10% to the labile, polar compounds, as judged by intensity of the colour with Folin-Ciocalteu reagent.

Both 18-hydroxyestrone and 18-hydroxyoestradiol-17 β were identified after incubations of 18-hydroxyestrone with rabbit liver slices. Labile polar compounds, similar to those described above, were also observed but not identified. It is noteworthy that 18-hydroxyoestradiol-17 α was not detected in these extracts. The extent of metabolism by rabbit liver slices appeared to be about half that judged for the rat liver. The extracts of the reagent blanks showed a single compound with the same properties as the substrate; the tissue blanks from both rat and rabbit liver were devoid of any phenolic substances.

Discussion

Two features of the chemistry of 18-hydroxylated oestrogens distinguish them from other oestrogens: (a) the action of strong alkali and acid and (b) the apparent ability of the 18-hydroxyl group to influence reactions at C-17.

(a) The formation of 18-noroestrone and formaldehyde as a result of the action of NaOH is unique to 18-hydroxyestrone, and was first described by Loke *et al.* (1959), who used a preparation from human pregnancy urine. In the present paper, 18-noroestrone has been identified as a product of NaOH treatment of authentic 18-hydroxyestrone. It is noteworthy that 18-noroestradiol-17 α and 18-noroestradiol-17 β were not formed after exposure of the 18-hydroxylated oestradiols to alkali. It is concluded therefore that NaOH can only be effective

Fig. 2. Mass spectrum of 18-hydroxyoestradiol-17 β
For details see the text.

Fig. 3. Mass spectrum of 18-hydroxyoestradiol-17 α
For details see the text.

Scheme 2.

Scheme 3.

if there is an oxo group at C-17. The mechanism of cleavage is envisaged as shown in Scheme 4.

A similar mechanism probably applies to the loss of formaldehyde from the primary β -ketols, methyl

hederagonate and icterogenin, as a result of alkali treatment (Barton & de Mayo, 1954) (Scheme 5).

Loke *et al.* (1959) suggested that H_2SO_4 treatment of 18-hydroxyoestrone could result in the formation

of 18-noroestrone and formaldehyde and that this might occur during the first step of the Kober reaction. As 18-noroestrone gives only a very weak Kober reaction (Loke *et al.*, 1959; Jones & Hähnel, 1969) this would explain the low Kober chromogenicity of 18-hydroxyoestrone. However, we were unable to find evidence for the formation of 18-noroestrone after treating 18-hydroxyoestrone with H_2SO_4 ; further, a mechanism for this reaction in H_2SO_4 is difficult to envisage. The relatively high chromogenicity of 18-hydroxyoestradiol-17 α and 18-hydroxyoestradiol-17 β would indicate that H_2SO_4 treatment of these compounds does not result in the formation of the corresponding 18-noroestradiols; this is supported by the fact that 18-noroestradiol-17 β has virtually no Kober chromogenicity (Jones & Hähnel, 1969). Thus a difference in the ability to form 18-nor derivatives does not appear to explain the difference in Kober chromogenicity between 18-hydroxyoestrone and the epimeric 18-hydroxyoestradiols. It is questionable then whether 18-hydroxylated oestrogens form their Kober chromogens by a similar mechanism to that proposed for oestradiol-17 β (Jones & Hähnel, 1969). It is also questionable whether migration of the methyl group

from C-13 to C-16 is always a necessary pre-requisite for a positive Kober reaction (Jones & Hähnel, 1969). This pre-requisite is not supported by the fact that 18-hydroxyoestrogens give a Kober reaction.

The property of oestradiol-17 α to form a chromogen in dilute H_2SO_4 (Haenni's Reagent B) was not found with 18-hydroxyoestradiol-17 α . Similarly the behaviour of 18-hydroxyoestradiol-17 α in phosphomolybdic acid differed from that of oestradiol-17 α . The differences between oestradiol-17 α and its 18-hydroxy derivative may be interpreted as either steric influence of the 18-hydroxyl group or a direct involvement of the 18-hydroxyl group in the colour reactions.

(b) The most convincing evidence for an influence of the 18-hydroxyl group on the reactions at C-17 has been its effect on the relative formation of 18-hydroxyoestradiol-17 α (30%) and 18-hydroxyoestradiol-17 β (70%) from 18-hydroxyoestrone by the action of NaBH_4 in methanol; this is to be compared with an almost 100% formation of oestradiol-17 β from oestrone (cf. Kirk & Hartshorn, 1968). Dauben *et al.* (1956) postulated that the observed stereochemical composition of products, resulting from reduction with complex hydrides, is a consequence of the interplay of two opposing factors, namely 'product development control' and 'steric approach control'. The former implies acceptance of the view that the factors affecting the relative stabilities of the products are also able to influence the energy levels of the respective transition states. This is derived from the observation that unhindered ketones are reduced to give predominantly the more stable product, i.e. the equatorial alcohol (Kirk & Hartshorn, 1968). The less stable axial alcohols predominate when the approach of the reducing agent along the quasi-axial direction is hindered by the presence of a *syn*-axial methyl group. Reduction of ring-D ketones may be subject to both 'steric and product development control'; normally, 17-ketones give only 17 β -alcohols as a result of nucleophilic attack from the less hindered α -direction (Kirk & Hartshorn, 1968). It is possible that the larger proportion of the α -epimer (18-hydroxyoestradiol-17 α) is due to an affinity of the NaBH_4 for the 18-hydroxyl group, which is sufficient to pre-determine a larger proportion of approaches from the β -direction. This would constitute 'steric approach control' executed through the agency of the 18-hydroxyl group.

Unlike NaBH_4 reduction, the enzymic reduction of 18-hydroxyoestrone by liver slices from rat and rabbit led exclusively to the formation of the 17 β epimer (Scheme 6). The absence of formation of 18-hydroxyoestradiol-17 α by rabbit liver slices was particularly notable in view of the description of a 17 α -hydroxy steroid oxidoreductase in that tissue

Scheme 6. Reactions of 18-hydroxyoestrone with rat and rabbit liver slices

—→, Reaction shown to occur; —|→, reaction shown not to occur; - - -→, reaction possibly occurring.

by Ball & Breuer (1970). In the present investigation, only the free steroid fractions were examined, which were estimated to contain approx. 60% of the incubated substrate. The 17-epimer could have been present in a water-soluble form. The polar, labile metabolites observed after initial chromatography had properties very similar to 2-hydroxylated derivatives (cf. Marks & Hecker, 1969). 2-Hydroxylase activity has been reported in rat (Adlercreutz, 1970) and rabbit (King, 1961) liver. No chromatographic evidence for the formation of 18-noroestrone from 18-hydroxyoestrone *in vitro* was found.

The identification of 18-hydroxyoestradiol-17 β as well as other more polar metabolites from

18-hydroxyoestrone *in vitro* raises the possibility of the endogenous existence of these metabolites.

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