7-HYDROXYLATION OF DEHYDROEPIANDROSTERONE BY RAT-LIVER HOMOGENATE

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SUMMARY

Among the products of the conversion of dehydroepiandrosterone in a rat-liver homogenate the following 7-oxygenated Δ^5-steroids were found: Δ^5-androsten-3β-ol-7,17-dione, Δ^5-androsten-3β,7α-diol-17-on and probably Δ^5-androsten-3β,7α,17β-triol.

The yield of 7-substituted steroids during the incubation increased by preheating of the homogenate, but not by addition of ATP or DPN. It is inhibited by Amphenone.

7-Hydroxylation was proved to be a normal subsidiary pathway of dehydroepiandrosterone degradation.

INTRODUCTION

The 7-hydroxylation of cholesterol by the action of *Proactinomyces roseum* was the first microbial hydroxylation of steroids reported[1]. Hydroxylation into position 7 was found also in other microorganisms and is encountered in saturated as well as unsaturated steroids with a 3-ketogroup or a 3β-hydroxyl group mostly into position 7β (see refs. 2–6), but a 7α-hydroxylation was also described[7,8]. Quite recently a 7α- and 7β-hydroxylation of dehydroepiandrosterone[9] by the action of *Rhizopus sp.* and *Fusidium sp.* was demonstrated.

Although microbial 7-hydroxylations of steroids are common, the introduction of the hydroxyl group into this position of C_{18}, C_{19} or C_{21} steroids by animal tissues was not studied hitherto. Only the 7-hydroxylation of cholesterol[10] and of bile acids in the liver was investigated in detail.

EXPERIMENTAL

Reference substances

Δ^5-Androsten-3β,7α-diol-17-one (7-OH-DHA) and its 7β-epimer were prepared by acetoxylation of dehydroepiandrosterone by the action of *tert.*-butylperacetate and alkaline hydrolysis of the acetate[11], m.p. 182–3°, $[\alpha]_D^{21}$ — 72°, resp. m.p. 212–4°, $[\alpha]_D^{21}$ + 65°. Δ^5-Androsten-3β,7α,17β-triol was prepared from 7-OH-DHA by reduction with $LiAlH_4$ and melted at 205°, $[\alpha]_D^{21}$ — 76.5°. $\Delta^{5,7}$-Androstadien-3β-ol-17-one was

Abbreviation: DHA, dehydroepiandrosterone.

obtained by dehydration of 7-OH-DHA with 2% HCl in 90% methanol and melted at 141°.

Preparation of the liver homogenate and incubation

Livers of females 150–200 g Wistar rats were weighed and homogenised in the Waring blendor in Ringer–Krebs phosphate at 4° (125 ml/10 g tissue). The homogenate with 0.02 *M* glucose was incubated with dehydroepiandrosterone, dissolved in 0.5 ml triethylene glycol; 1 mg of steroid was used for 2 g of liver tissue. The incubation took place at 38°, oxygen was bubbled through the mixture. The time of incubation was 1 h, unless stated otherwise. If explicitly stated, DPN was added (34 mg/100 ml), ATP (20 mg/100 ml) and Amphenone B in a final concentration of $5 \cdot 10^{-4}$ *M*.

Treatment of the incubation mixture

After the incubation was completed, the double volume of ethyl acetate was added to the mixture and after shaking, the mixture was left overnight. After separation of the coagulated proteins and the aqueous layer the ethyl acetate extract was washed with water, dried over anhydrous sodium sulphate and the ethyl acetate was distilled off *in vacuo*.

Purification and analysis of extract

The evaporated residue dissolved in a small amount of chloroform was subjected to chromatography on alumina of activity II in a spread-layer on a glass plate[11–13] using benzene with 5% ethanol as the mobile phase. The zones corresponding to the authentic samples of steroids were separated and eluted with 10 ml ethanol. The eluates were evaporated and applied to Whatman No. 1 paper in such manner that onto Bush's system an amount corresponding to 300 μg of incubated DHA was transferred, double the amount was added to the systems with glycol stationary phase. The mobilities of the investigated steroids in the chromatographic systems used are listed in Table I and their chromogenic properties in Table II.

The chromatograms developed in Bush system were detected by means of antimony trichloride reagent (saturated solution of $SbCl_3$ in chloroform with acetanhydride

TABLE I

CHROMATOGRAPHIC MOBILITIES OF 3β-HYDROXY-Δ^5-STEROIDS

Steroid	*Solvent system* (R_F values)*					
	B–E	*B 5*	*P–T*	*C–T*	*B–T*	*Alumina*
Δ^5-androsten-3β-ol-17-one (DHA)	0.89	0.93	0.22	0.62	0.66	0.70
Δ^5-androsten-3β,7α-diol-17-one (7-OH-DHA, I)	0.42	0.42	0.01	0.04	0.10	0.28
Δ^5-androsten-3β-ol-7,17-dione (II)	0.64		0.05	0.12	0.36	0.55
Δ^5-androsten-3β,7α,17β-triol (III)	0.15	0.08	0.00	0.00	0.02	0.13
$\Delta^{5,7}$-androstadien-3β-ol-17-one		0.85	0.16	0.52	0.61	0.68

* Whatman No. 1 paper, chromatographed at 20°. Solvent systems: B–E butyl acetate–ethylene glycol[14]; B 5 Bush B5; P–T petroleum ether–triethylene glycol[15]; C–T carbon tetrachloride–triethylene glycol[15]; B–T benzene–triethylene glycol[15] alumina: spread-layer chromatography on alumina activity II, mobile phase benzene with 5% ethanol.

TABLE II

CHROMOGENIC PROPERTIES OF 3β-HYDROXY-Δ^5-STEROIDS

Steroid	Reagent*				Chromatographic fraction (alumina)
	PTA	$SbCl_3$	Z	NaOH	
Δ^5-androsten-3β-ol-17-one	Orange	Red	+	—	29–35
Δ^5-androsten-3β,7α-diol-17-one	Blue	Blue	+	—	46–48
Δ^5-androsten-3β-ol-7,17-dione	Orange	—	+	+	36–38
Δ^5-androsten-3β,7α,17β-triol	Blue	Blue	—	—	68–72
$\Delta^{5,7}$-androstadien-3β-ol-17-one	Blue	Blue	+	—	

* PTA phosphotungstic acid; $SbCl_3$, antimony trichloride reagent (saturated $SbCl_3$ in chloroform–acetanhydride 4 : 1); Z, Zimmermann reagent (alkaline alcoholic solution of *m*-dinitrobenzene); NaOH, ultraviolet fluorescence after 10% NaOH in 60% methanolic spray and heating to 90°.

4:1) and the amount of Δ^5-7-hydroxysteroid was assessed by comparison of the characteristic blue coloration with the colour of a series of standard samples of 7-OH-DHA developed simultaneously.

The chromatograms developed in systems with glycol as the stationary phase[14,15] were evaluated colorimetrically after detection with ZIMMERMANN's reagent by the method described previously[16,17] against an aliquot portion of homogenate incubated without addition of DHA.

Preparative hydroxylation by liver homogenate

The homogenate from 35 g liver was incubated in Ringer–Krebs phosphate with glucose for 1 h at 38° with 220 mg DHA. The ethyl acetate extract was fractionated by chromatography on a column of alumina activity II (50 g, height of column 15 cm) by gradient elution with benzene with increasing amounts of ethanol up to 20%. One hundred fractions of 7 ml were collected, aliquot portions were evaporated and the steroid content was assayed colorimetrically after ZIMMERMANN's reaction, after reaction with $SbCl_3$ (at 665 mμ) and spectrophotometrically at 240 mμ in ethanol. The composition of the fractions was investigated by paper chromatography. The fractions, in which the steroids were found are shown in Table II.

The formation of 7-OH-DHA in the model medium

EDTA–phosphate buffer was used, EDTA–phosphate buffer with ferrous sulphate and EDTA–phosphate buffer with Fe^{2+} and ascorbic acid[18,19]. DHA was added in a solution of triethylene glycol.

RESULTS

During the incubation of DHA with the rat-liver homogenate in addition to known product of liver conversion of this steroid[20–22] metabolites not yet described were formed of which one (II) was an α,β-unsaturated ketone, the other (I) gave a typical blue coloration with $SbCl_3$, characteristic for Δ^5-7-hydroxysteroids respectively $\Delta^{5,7}$-diens. Sometimes a more polar substance (III) with the same typical reaction was found.

Metabolite I was identified as Δ^5-androsten-3β,7α-diol-17-one: it had the same

mobility in three chromatographic systems on paper and on alumina and the same staining characteristic as the authentic sample of 7-OH-DHA. On dehydration with 2% HCl in 90% methanol resulted mainly a product the chromatographic behaviour of which was identical with that of $\Delta^{5,7}$-androstadien-3β-ol-17-one. After reduction with $LiAlH_4$ the resulted steroid did not differ chromatographically from Δ^5-androsten-3β,7α,17β-triol.

The metabolite I obtained by preparative liver hydroxylation of DHA in amount of 2 g melted at 179° and did not produce a depression of the melting point with the authentic sample. The optical rotation of the fractions with metabolite I was markedly negative which in addition with the rate of formation of $\Delta^{5,7}$-dien suggests the 7α-position of the hydroxyl function.

The metabolite II was an α,β-unsaturated ketone, m.p. 245-7°; with the authentic sample of Δ^5-androsten-3β-ol-7,17-dione it did not produce a depression of the melting point and had the same chromatographic behaviour and staining characteristic. By reduction with $LiAlH_4$ it produced a chromatographically identical substance with Δ^5-androsten-3β,7β,17β-triol. Metabolite II was formed also after the incubation of 7-OH-DHA.

The metabolite III had all chromatographic and chromogenic properties of Δ^5-androsten-3β,7α,17β-triol; it proved, however, impossible to obtain it in crystalline form. Metabolite III was obtained after incubation of DHA as well as after incubation of 7-OH-DHA.

The time course of the formation of 7-OH-DHA and of the 7-ketoderivative is shown in Fig. 1. Dehydroepiandrosterone disappears rapidly from the reaction medium; the maximal formation of the 7-hydroxy-derivative occurs, under the conditions described, after 40–60 min, the maximum formation of Δ^5-androsten-3β-ol-7,17-dione is retarded by about 20 min. After the incubation of 7-OH-DHA with

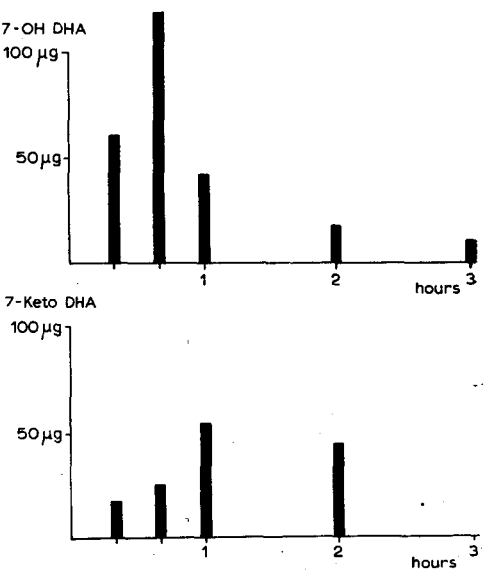


Fig. 1. Influence of time on formation of 7-oxygenated-3β-hydroxy-Δ^5-steroids in rat-liver homogenate.

TABLE III

FORMATION OF 7-OXYGENATED 3β-HYDROXY-Δ^5-STEROIDS IN VARIOUS MEDIA

	Medium	*Per cent substrate oxygenated to*	
		7-OH-DHA	*Δ^5-androsten-3β-ol-7,17-dione*
	Liver homogenate	0.0	0.0
	Liver homogenate + DHA	1.2–2.3	
	Liver homogenate preheated + DHA	4.8–6.2	
	Liver homogenate + DHA + DPN	0.1–0.3	
	Liver homogenate + DHA + ATP	0.2–0.4	
	Liver homogenate + 7-OH-DHA	22.5	20.5
model system	Phosphate buffer + DHA	0.0	
model system	EDTA–phosphate buffer + DHA	0.0	
model system	EDTA–phosphate buffer + DHA + Fe^{2+}	0.0	
model system	EDTA–phosphate buffer + DHA + ascorbic acid	0.0	
model system	EDTA–phosphate buffer + DHA + Fe^{2+} + ascorbic acid	0.6–0.8	

liver homogenate after 1 h 22.5% of unchanged steroid was found, 22.0% of the 7-ketoderivative, the remainder were unidentified substances.

From the results presented in Table III it appears that in the liver tissue there is no 7-OH-DHA nor any substance simulating its presence before the incubation of DHA. Dehydroepiandrosterone is not autooxidised to any marked extent to 7-OH-DHA. The amount of converted steroid is not increased by DPN nor by ATP; the 7-hydroxylation is completely inhibited by the addition of Amphenone in a concentration of $5 \cdot 10^{-4}$ *M*. In this case after 1 h incubation considerable amounts of unaltered DHA were found. The results mentioned were obtained with the same liver homogenate; otherwise, however, a remarkable variability between different preparations of liver homogenate was observed: only rarely were much greater amounts of 7-hydroxylated metabolites found, in several instances the conversion of DHA to 7-OH-DHA was, on the other hand, somewhat lower than the values listed in the Table III.

In the media used in the absence of homogenised liver tissue the 7-hydroxylation of dehydroepiandrosterone took place only in EDTA–phosphate buffer with ferrous ions and ascorbic acid, while autooxidation was not observed in the other media.

Interesting is the increased yield of 7-hydroxylated steroid after heating the homogenate before incubation to 75°; an activation of this type was found also in other enzymic processes, which can be also induced in a medium containing Fe^{2+} and ascorbic acid under aerobic conditions[23,24].

DISCUSSION

In the urine of a patient with adrenal carcinoma Δ^5-androsten-3β-ol-7,17-dione[25] was found as well as Δ^5-androsten-3β,7α-diol-17-one[26] in addition to their 16α-hydroxy-derivatives[26]. Δ^5-Androsten-3β-ol-7,17-dione was later reported as a normal metabolite in human urine[27] and the 7α-hydroxyderivative of DHA was found after the

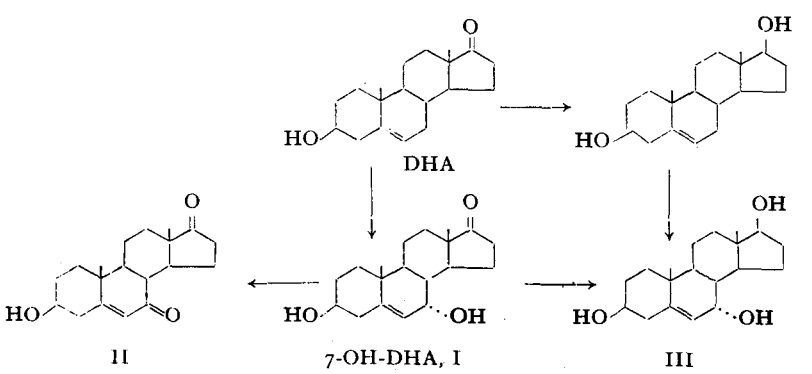
administration of the precursor in a normal man[28] and in rats[29] and even as a normal metabolite of endogenous origin in human urine[29].

These facts suggest the probable tissue 7-hydroxylation of Δ^5-steroids, which, however, was not confirmed so far by other means than by isolation of 7-substituted steroids from urine. The site of 7-hydroxylation was anticipated to be in the liver, though first the hypothesis was expressed that it might take place in the adrenals in view of DHA excretion and the excretion of its 7-ketoderivative after the administration of Amphenone B which might suggest the biosynthesis of 7-substituted steroid independently on the DHA production[25,30]. The peripheral origin of 7-OH-DHA is, however, confirmed by the course of the 7-hydroxylation in rat-liver homogenates under aerobic conditions. As compared with the possibilities of studying 7-hydroxylation of cholesterol in the liver[12], the situation is more clear because no apparent autooxidation into position 7 takes place under conditions of a blank experiment. During the isolation of products of liver conversion the sensitivity of Δ^5-7-hydroxysteroids at lower pH, particularly those with the pseudoaxial 7α-hydroxygroup, must be taken into account, as the labile allyl hydroxyl group is easily split off and $\Delta^{5,7}$-diene is formed and a further destruction of the molecule takes place, as is usually the case when the substance is isolated from urine.

SCHNEIDER AND MASON[20] investigated the conversion of DHA by liver tissue slices and found a reduction of the 17-ketogroup and the formation of Δ^5-androsten-3β,17β-diol (43–69% in 3–6 h by rabbit-liver slices), 16α-hydroxylation and the formation of Δ^5-androsten-3β,16α,17β-triol (2.4–8.9% conversion) and the formation of two not identified substances, of which one was an α,β-unsaturated ketone with a melting point 224–6°, acetate m.p. 175–6° (1% conversion). This substance could be identical with Δ^5-androsten-3β--ol-7,17-dione the formation of which was revealed in a similar quantitative ratio. UNGAR, MILLER AND DORFMAN[21] confirmed some of the findings of SCHNEIDER AND MASON and found also a small amount of Δ^4-androsten-3,17-dione. Recently[22] six metabolites of DHA were found after perfusion with dog liver, one of them being a polar unidentified compound, probably hydroxylated in the position 16 or 7.

Two 7-oxygenated steroids, which were presently found in addition to the 7-ketoderivative of DHA, namely Δ^5-androsten-3β,7α-diol-17-one and Δ^5-androsten-3β, 7α, 17β-triol were now first identified as a conversion product of DHA in rat-liver homogenate. Their formation can be illustrated by the following scheme:

Scheme I



While the disappearance of DHA from the incubation mixture is very rapid, the conversion of 7-OH-DHA to the corresponding 7-ketoderivative proceeds more slowly. In view of the rapid conversion of DHA to androstendiol there is also the possibility of 7-hydroxylation of this steroid which can be formed also from 7-OH-DHA by reduction of its 17-ketogroup. The concurrent or subsequent 16α-hydroxylation of these products cannot be excluded, but was not investigated.

The enzymic system mediating the 7-hydroxylation of Δ^5-steroids in the liver is not activated by DPN nor by ATP, it can be, however, activated by preheating of the incubation mixture. 7-Hydroxylation takes place also under aerobic conditions in a medium containing ferrous ions and ascorbic acid. In a similar system hydroxylation of various aromatic compounds[18,19] and deiodation of iodotyrosine and iodothyronine[23] was proved. It is interesting that the proved enzymic course of the above reactions in liver tissue has some features common with the observed 7-hydroxylation of Δ^5-steroids. Thus for instance was found[24] that deiodases of thyroxine are activated when the liver tissue is preheated for several minutes to 100° and that the system is not activated by DPN, TPN, DPNH or TPNH.

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