The Intermediary Role of a 19-Oxoandrogen in the Biosynthesis of Oestrogen

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Previous work in a number of Laboratories has established that the biological conversion of androstenedione (I) into oestrone (V) occurs through the involvement of a 19-hydroxy compound (II) (Meyer, 1955; Ryan, 1959; Hayano, Longchampt, Kelly, Gual & Dorfman, 1960; Longchampt, Gual, Ehrenstein & Dorfman, 1960; Breuer & Grill, 1961; Morato, Hayano, Dorfman & Axelrod, 1961; Axelrod & Goldzeiher, 1962; Wilcox & Engels, 1965). The present work was undertaken to study the nature of the metabolic processes involved in the formation of oestrone from the 19-hydroxy compound. The results described below show that the conversion of 19-hydroxyandrostenedione (II) into oestrone takes place in at least two stages, the first being the formation of a 19-oxo intermediate (IV). Attempts to elucidate the mechanism of this reaction, (II) $\rightarrow$ (IV), reveal that the process is dependent on the involvement of NADPH and oxygen. This requirement allows one to conclude that the oxidation of the primary alcohol group in compound (II) to an aldehyde group in compound (IV) must proceed through a hydroxylated intermediate (III). The further enzymic conversion of the aldehyde (IV) into oestrone once again requires NADPH and oxygen.

For our mechanistic work we needed certain 19-oxygenated steroids; two of these, 19-hydroxydehydroepiandrosterone (VI) and 19-oxodehydroepiandrosterone (VII), labelled with tritium at the 16-position, were synthesized as described in the Experimental section. When 19-hydroxydehydroepiandrosterone (VI) and 19-oxodehydroepiandrosterone (VII) were separately incubated under anaerobic conditions with a 105 000g microsomal pellet, prepared from human full-term placenta (Ryan, 1959), in the presence of NADP⁺, a ready conversion into the corresponding conjugated ketones, 19-hydroxyandrostenedione (II) (Expt. 3, Table 1) and 19-oxoandrostenedione (IV) (Expt. 8, Table 1) occurred. The substitution of NAD⁺ for NADP⁺ in the preceding incubations gave the same products in similar yields (Expts. 7 and 12 respectively).

When the biological conversion of 19-oxodehydroepiandrosterone (VII) was carried out in the presence of NADP⁺ but under aerobic conditions, oestrone was formed in 15% yield (Expt. 10).

However, under identical conditions 19-hydroxydehydroepiandrosterone (VI) gave no significant amount of oestrone (about 2%), but a compound with the properties of 19-oxoandrostenedione (IV) was formed in 5% yield of the radioactivity incubated (Expt. 5).

Chromatographic and degradative experiments described in the Experimental section unambiguously established that the material was indeed 19-oxoandrostenedione (IV). It is to be noted in the above incubations a limited amount of NADPH must be generated as a result of the enzymic oxidation of the 3 β -hydroxyl group by reactions of the types 1 and 2 (Scheme 1) to the conjugated ketone. We believe that the NADPH formed in these reactions is used in both the conversions (II) $\rightarrow$ (IV) and (IV) $\rightarrow$ (V). This conclusion on the involvement of NADPH and oxygen in these conversions is further substantiated by the following experiments.

19-Oxodehydroepiandrosterone (VII) was incubated with placental microsomes in the presence of NADP⁺ and oxygen as in Expt. 10, but in this case the newly formed NADPH (reaction 2, Scheme 1) was rapidly removed by the inclusion of GSSG and glutathione reductase in the incubation mixture. Under these conditions no significant conversion of the compound (VII) into oestrone occurred (Expt. 11). On the other hand, a generous supply of NADPH in similar incubation conditions but without GSSG and glutathione reductase gave a high conversion of both (VI) and (VII) into oestrone (Expts. 4 and 9).

To demonstrate directly the conversion of 19-hydroxyandrostenedione (II) into 19-oxoandrostenedione (IV) under conditions of oestrone biosynthesis, the former compound (II) labelled with tritium at the 16-position was incubated with NADP⁺ and placental microsomes under aerobic conditions and non-radioactive 19-oxodehydroepiandrosterone (VII) was added to the incubation mixture to supply a steady but limited amount of NADPH as in reaction 2 (Scheme 1). The experiment had the added advantage that the non-radioactive aldehyde formed by the reaction (VII) $\rightarrow$ (IV) (reaction 2, Scheme 1) could act as a trap for the radioactive intermediate. In the experiment about 3% of the radioactivity of

Table 1. *Metabolic conditions for the conversion of 16-³H₂-labelled 19-oxygenated androgens into oestrone and its precursors*

Incubations were carried out as described in the Experimental section. In Expts. 1, 2, 4, 9, 14, 16 and 17, NADPH was generated by the addition of 8mg. of glucose 6-phosphate and 0.5 unit of glucose 6-phosphate dehydrogenase to the incubation mixture. In Expts. 3, 5, 6, 7, 8, 10, 12 and 13, a limited amount of NADPH or NADH was formed due to the enzymic oxidation of the 3-hydroxyl group of the substrates (as in reactions 1 and 2, Scheme 1). In Expt. 15 a limited amount of NADPH was generated by including in the incubation mixture non-radioactive 19-oxodehydroepiandrosterone (cf. reaction 2, Scheme 1). The 19-oxoandrostenedione formed in this reaction acted as a 'trap' as described in the text. In Expt. 11 any reduced cofactor produced by enzymic oxidation of the 3 β -hydroxyl group of the radioactive substrate is rapidly reoxidized by the inclusion of 10mg. of GSSG and 3 units of glutathione reductase.

Substrate (200 μ g.)	Expt. no.	Cofactor	O ₂ or N ₂	Products (% yield $\dagger$)
[4- ¹⁴ C]Androstenedione	1	NADPH*	O ₂	Oestrone (20%)
[4- ¹⁴ C]Testosterone	2	NADPH*	O ₂	Oestrone (15%)
19-Hydroxy[16- ³ H]dehydro-epiandrosterone (VI)	3	NADP ⁺	N ₂	(II) (50%)
	4	NADPH*	O ₂	Oestrone (40%)
	5	NADPH $\dagger$	O ₂	(II) (50%), (IV) (5%), oestrone (2%)
	6	NADH $\dagger$	O ₂	(II) (50%)
	7	NAD ⁺	N ₂	(II) (50%)
19-Oxo[16- ³ H]dehydro-epiandrosterone (VII)	8	NADP ⁺	N ₂	(IV) (50%)
	9	NADPH*	O ₂	Oestrone (30%)
	10	NADPH $\dagger$	O ₂	(IV) (30%), oestrone (15%)
	11	NADP ⁺	O ₂	(IV) (40%), oestrone (1%)
	12	NAD ⁺	N ₂	(IV) (50%)
	13	NADH $\dagger$	O ₂	(IV) (50%)
19-Hydroxy[16- ³ H]androstenedione (II)	14	NADPH*	O ₂	Oestrone (50%)
	15	NADPH $\dagger$	O ₂	(IV) (3%), oestrone (1.2%)
19-Oxo[16- ³ H]androstenedione (IV)	16	NADPH*	O ₂	Oestrone (50%)
	17	NADPH*	N ₂	Oestrone (2%)

* NADP⁺ reduced by glucose 6-phosphate and glucose 6-phosphate dehydrogenase.

$\dagger$ NADP⁺ reduced by 3 β -hydroxyl group of steroid substrate.

$\ddagger$ Products expressed as percentage yield of radioactivity incubated.

compound (II) was incorporated into compound (IV) (Expt. 15).

The cumulative evidence presented above suggests the intermediary role of 19-oxoandrostenedione in the biological conversion of 19-hydroxyandrostenedione into oestrone. The requirement for NADPH and oxygen would be consistent with the involvement of a hydroxylated intermediate of the type (III) in the conversion of compound (II) into compound (IV).

Though the involvement of 19-oxoandrostenedione as an intermediate in oestrone biosynthesis has been advocated before (Hayano *et al.* 1960; Morato *et al.* 1961; Axelrod & Goldzeiher, 1962), as far as we are aware this is the first demonstration of its formation under the conditions of oestrogen biosynthesis.

To summarize, it is shown that the conversion of 19-hydroxyandrostenedione (II) into oestrone (V)

occurs in at least two stages both requiring NADPH and oxygen, the first stage being the formation of 19-oxoandrostenedione (IV). The accumulation of the compound (IV) under the conditions of oestrogen biosynthesis can best be demonstrated when the conversion is carried out in the presence of a limited supply of NADPH.

Our results, in conjunction with those of Townsley & Brodie (1968), may be interpreted to suggest that in the further conversion of the aldehyde (IV) into oestrone another hydroxylated intermediate may be involved.

Experimental. (a) Preparation of microsomes and incubation procedure. Placental microsomes were prepared from human full-term placenta by the method of Ryan (1959) and stored as portions equivalent to approx. 30g. wet wt. of placenta at -16° in 2.5ml. of 0.05M-sodium phosphate buffer, pH 7.1.

Scheme 1.

Each incubation mixture contained 2.5 ml. of 0.05 M-sodium phosphate buffer, pH 7.1, 200 μ g. of substrate dissolved in either 0.1 ml. of methanol or 0.1 ml. of propylene glycol, 4 mg. of NADP⁺ or NAD⁺, as indicated in Table 1, and 2.5 ml. of the microsomal preparation, and was incubated at 37° for 2 hr.

(b) Synthesis of 19-oxygenated androgens. 3 β -Acetoxy-19-oxo- and 3 β -acetoxy-19-hydroxydehydroepiandrosterone were synthesized by the method described by Akhtar & Barton (1964). The corresponding 3- β -hydroxy (16-³H-labelled) compounds were prepared by exchange with methanolic 5% (w/v) KOH containing tritiated water (50 mc/ml.) (Akhtar, Parvez & Hunt, 1968).

(c) Extraction and purification of steroids in the incubation mixture. The incubations were terminated and the steroids extracted as described by Wilcox & Engels (1965) and applied to thin-layer plates (silica gel GF₂₅₄). The plates were first run in benzene-ether (1:2, v/v), which resolved the steroids in three main groups: A, oestrone (R_F 0.65–0.75); B, 19-oxoandrostenedione and oestradiol (R_F 0.35–0.45); C, 19-hydroxyandrostenedione, 19-oxodehydroepiandrosterone and 19-hydroxydehydroepiandrosterone (R_F 0.00–0.20). The band B was rechromatographed on a thin layer with methanol-chloroform (1:19, v/v) to give 19-oxo-

androstenedione (R_F 0.55) and oestradiol (R_F 0.40). The radioactive material from the thin-layer chromatogram was eluted with methanol-chloroform (1:4, v/v).

(d) Proof of the structure of the 19-oxoandrostenedione (IV) intermediate from Expt. 5. The fraction believed to contain 19-oxoandrostenedione (radioactivity 12 720 counts/min.) was diluted with carrier (1 mg.) and dissolved in methanol (2 ml.). The solution was cooled to 0° and then reduced with NaBH₄ (0.5 mg.). The reaction mixture was spotted on a thin-layer plate and developed in methanol-chloroform (1:19, v/v) and 17,19-dihydroxyandrost-4-en-3-one, which was formed in 35% yield, contained 4270 counts/min. This on dilution with carrier (43 mg.) was crystallized to constant specific radioactivity, retaining 100% of the radioactivity eluted from the plate (82.87 counts/min./mg.; 25 226 counts/min./m-mole). The 17,19-dihydroxyandrost-4-en-3-one (25 mg.) was acetylated for 16 hr. in acetic anhydride plus pyridine and the product crystallized. The first crystallization gave material of specific radioactivity 58.60 counts/min./mg. (22 766 counts/min./m-mole) (90.24% retention of radioactivity); the second crystallization gave material of specific radioactivity 60.3 counts/min./mg. (23 427 counts/min./m-mole) (92.9% retention of radioactivity).

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