Biosynthesis of 3β-hydroxy-5,7-pregnadien-20-one by the horse fetal gonad

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The production of equilin and the other ring B-unsaturated estrogens by the pregnant mare is anomalous in that they are biosynthesised by a cholesterol-independent pathway. Fetal horse gonads were incubated with tritiated sodium acetate and radiochemically pure 3β-hydroxy-5,7-pregnadien-20-one and 3β-hydroxy-5,7-androstadien-17-one were isolated. A fetal gonad–placental system is proposed for equilin production, 3β-hydroxy-5,7-pregnadien-20-one being a precursor for 3β-hydroxy-5,7-androstadien-17-one in the fetal gonad and the latter being the precursor of equilin in the placenta. The nature of the possible precursor of 3β-hydroxy-5,7-pregnadien-20-one is discussed.

Equilin *Biosynthesis* *Precursor* *Horse* *Fetal gonad*

1. INTRODUCTION

The blood and urine of pregnant mares have high concentrations of estrogens; e.g., estrone (I) and also ring B unsaturated estrogens; e.g., equilin (II). It has been shown that whereas estrone is formed by the classical triterpene (C 30) pathway of steroid biosynthesis equilin is biosynthesised by an alternative route which avoids both squalene and cholesterol [1,2]. It has been proposed that this alternative route could be a sesterterpene (C 25) pathway [3,4].

A prerequisite for the formation of ring B unsaturated estrogens by human and horse placenta in vitro is the presence of a 7(8) double bond in the C 19 precursor [5,6,7]. The horse fetal gonad is implicated in the provision of substrates for subsequent estrogen formation in the placenta [8,9] and it has recently been shown that the gonadal tissue can biosynthesize 3β-hydroxy-5,7-androstadien-17-one (III) from radioactive acetate in vitro [4]. From these findings it was proposed that, in the pregnant mare, equilin is produced in the placenta by aromatisation of 3β-hydroxy-5,7-androstadien-17-one which is biosynthesised in the fetal gonad. This proposal is analogous to the fetal adrenal–placental system for estrogen biosynthesis in human pregnancy [10].

The possibility has now been examined that other 5,7-dienes could be produced by the horse fetal gonad and this report describes the biosynthesis of 3β-hydroxy-5,7-pregnadien-20-one (IV).

2. EXPERIMENTAL

3β-Hydroxy-5,7-pregnadien-20-one and 3β-hydroxy-5,7-androstadien-17-one were synthesised as in [11]. Thin-layer chromatography (TLC) was carried out on 0.25 mm thick layers of Kieselgel HF 254/366 (Merck). The solvent systems used were: cyclohexane–acetone ((A) 3:2, v/v; (B) 19:1, v/v); cyclohexane–ethyl acetate ((C) 2:1, v/v; (D) 9:1, v/v); toluene–diethyl ether ((E) 2:1, v/v; (F) 3:1, v/v); toluene–ethyl acetate ((G) 2:1, v/v); petroleum ether (b.p. 60–80°C)–ethyl acetate ((H) 9:1, v/v); dichloromethane–acetone ((I) 49:1, v/v). High pressure liquid chromatography (HPLC) was carried out on a Waters radial com-

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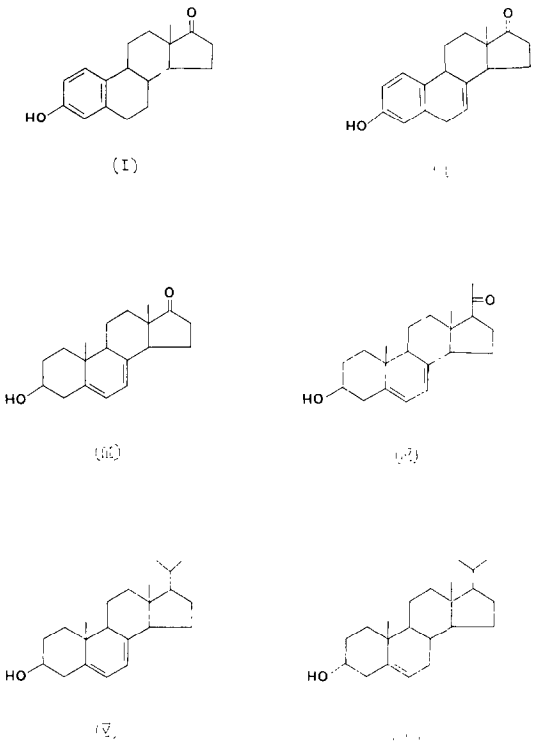

pression C-18, 10 μM column using 10% water in methyl alcohol as the mobile phase. Procedures for reduction, acetylation and hydrolysis of steroids were as described in [12].

2.1. *Experiment 1*

Fetal gonad (2.7 g from an 8-month fetus) was finely chopped, suspended in Eagles medium (10 ml) containing sodium [^{3}H]acetate (37 MBq, spec. act. 11.1 MBq/mmol) and 3β-hydroxy-5,7-pregnadien-20-one (0.16 μmol) and incubated at 37°C for 3 h in an atmosphere of oxygen/carbon dioxide (95:5). Ethyl alcohol (10 ml) was added and the mixture extracted with diethyl ether (3 × 50 ml). The organic phases were combined, washed with water and the solvent evaporated leaving a residue which was separated into a sterol and steroid fraction by TLC in system A.

The steroid fraction was acetylated, chromatographed in system E and then on a silver nitrate/silica gel plate [4] in solvent C which separated 3β-hydroxy-5,7-pregnadien-20-one acetate, 3β-hydroxy-5,7-androstadien-17-one acetate and pregnenolone-dehydroepiandrosterone (DHA) acetates. 3β-Hydroxy-5,7-pregnadien-20-one acetate was further purified by TLC in system F, then by HPLC, diluted with carrier steroid (70 μmol) and crystallized to constant specific radioactivity. Radiochemical purity was confirmed by further recrystallizations after converting to derivatives (table 1). 3β-Hydroxy-5,7-androstadien-17-one was purified similarly and crystallized (table 1).

Pregnenolone and DHA acetates were separated in system F, and both chromatographed in systems I and G, carrier steroid (150 μmol) added and crystallized to constant spec. act. (table 1).

2.2. *Experiment 2*

Fetal gonadal tissue (2.5 g) was homogenised and incubated in 0.1 mM phosphate buffer (pH 7.4, 10 ml) containing nicotinamide (30 mM), magnesium chloride (4 mM), reduced glutathione (8 mM), glucose-6-phosphate (6 mM) and NADP (0.1 mM) [3]. The substrates were [4-^{14}C]cholesterol (185 kBq, spec. act. 1.85 GBq/mmol) and 23,24-dinor-[7α-^{3}H]-5-cholen-3β-ol (VI) (1.48 MBq, spec. act. 48 GBq/mmol) [3] and the incubation was carried out at 37°C for 2 h in air. The incubation was extracted and DHA isolated and crystallized as above (table 2).

3. RESULTS AND DISCUSSION

The isolation and rigorous purification of 3β-hydroxy-5,7-pregnadien-20-one (IV) by formation of derivatives and recrystallization demonstrates that the fetal horse gonad can biosynthesize this 21-carbon (C 21) 5,7-diene from radioactive acetate in vitro (incorporation 0.001%) (table 1). The 19-carbon (C 19) 5,7-diene, 3β-hydroxy-5,7-androstadien-17-one (III) was also produced (incorporation 0.005%) (table 1) confirming our previous finding [4].

Pregnenolone and DHA were also isolated from this incubation (table 1). In the classical biosynthetic pathway pregnenolone (C 21) is a precursor of DHA (C 19) and it is considered possible, by analogy, that 3β-hydroxy-5,7-pregnadien-20-one (C 21) is a precursor of 3β-hydroxy-5,7-androstadien-17-one (C 19). It is therefore suggested that, in this tissue, there is a 5,7-diene pathway operating in parallel to the classical pathway.

Table 1

Radiochemical purity of steroids isolated from expt 1

Steroid	Derivative formed	Spec. act.[a] dpm/μmol (SD)
3β-Hydroxy-5,7-pregnadien-20-one	–	3.43×10^5 (0.02)
	3β-Hydroxy-5,7-pregnadien-20-one acetate	3.45×10^5 (0.04)
	5,7-Pregnadiene-3β,20β-diol 3-acetate	3.46×10^5 (0.08)
	5,7-Pregnadiene-3β,20β-diol diacetate	3.56×10^5 (0.04)
3β-Hydroxy-5,7-androstadien-17-one	–	–
	3β-Hydroxy-5,7-androstadien-17-one acetate	6.95×10^5 (0.15)
	5,7-Androstadiene-3β,17β-diol 3-acetate	7.41×10^5 (0.06)
Pregnenolone (3β-Hydroxy-5-pregnen-20-one)	–	–
	3β-Hydroxy-5-pregnen-20-one acetate	1.51×10^4 (0.07)
	5-Pregnene-3β,20β-diol 3-acetate	1.50×10^4 (0.08)
Dehydroepiandrosterone (3β-Hydroxy-5-androsten-17-one)	–	–
	3β-Hydroxy-5-androsten-17-one acetate	1.43×10^5 (0.07)
	5-Androstene-3β,17β-diol diacetate	1.32×10^5 (0.04)

[a] The spec. act. were judged to be constant when 3 crops agreed to within ± 5%. For brevity only the means and standard deviations of the spec. act. of each derivative are shown

Table 2

Radiochemical purity of dehydroepiandrosterone isolated from expt 2

Steroid	Derivative formed	Spec. act. dpm/μmol (SD)		$^3H/^{14}C$
		Tritium	Carbon^{-14}	
Dehydroepiandrosterone (3β-Hydroxy-5-androsten-17-one)	3β-Hydroxy-5-androsten-17-one acetate	3.37×10^4 (0.20)	9.77×10^4 (0.16)	0.35
	5-Androstene-3β,17β-diol 3-acetate	3.65×10^4 (0.06)	9.54×10^4 (0.04)	0.38
	5-Androstene-3β,17β-diol diacetate	3.89×10^4 (0.04)	9.88×10^4 (0.12)	0.39

C *x*-5,7-diene⟶

C 21-5,7-diene⟶C 19-5,7-diene

3β-hydroxy-5,7-pregnadien-20-one	3β-hydroxy-5,7-androstadien-17-one

It has been suggested that this 5,7-diene pathway could be a sesterterpene pathway [3,4], with 23,24-dinor-5,7-choladien-3β-ol (V), analogous to 7-dehydrocholesterol on the classical pathway, being the appropriate intermediate. 23,24-Dinor-5-cholen-3β-ol (VI) is also a possible intermediate on the sesterterpene pathway and it has been shown to be converted to steroid hormones by canine and bovine adrenals [3,13] and by two human corticosterone secreting adrenal adenomata [14]. DHA containing both ^3H and ^{14}C was isolated when tritiated 23,24-dinor-5-cholen-3β-ol and ^{14}C-labelled cholesterol were incubated with this tissue (table 2). The presence of ^{14}C in the DHA confirmed the previous finding that cholesterol is converted to DHA [4] and the ^3H shows that the horse

fetal gonad can convert the 5-ene, 23,24-dinor-5-cholen-3β-ol (VI) to DHA. That is, this tissue can utilise a material with the 23,24-dinorcholane carbocyclic system to produce steroids which are usually thought to be derived from cholesterol. It is hoped to test whether the 5,7-diene, 23,24-dinor-5,7-choladien-3β-ol (V) will yield 3β-hydroxy-5,7-androstadien-17-one (III) with the material reported in this paper 3β-hydroxy-5,7-pregnadien-20-one (IV) as an intermediate.

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