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IN VIVO BIOSYNTHESIS OF STEROIDS FROM TYROSINE

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SUMMARY

A mixture of [^{14}C]-tyrosine and [^{3}H]-dehydroepiandrosterone was injected into a horse fetus intramuscularly during laparotomy, after which maternal urine was collected for five days. Estrone and equilin were isolated from the phenolic fraction and their radiochemical purity established. Only estrone contained both [^{3}H] and [^{14}C], while equilin contained only [^{14}C]. From the neutral fraction [^{14}C]-labeled 3β-hydroxy-5α-pregnan-20-one, 5α-pregnane-3β, 20β-diol and 5α-pregnane-3β, 20α-diol were isolated. These results demonstrate that an aromatic amino acid such as tyrosine can serve as a carbon source for steroids in the pregnant mare. Since both neutral and phenolic steroids were formed from [^{14}C]-tyrosine it is unlikely that preformed benzenoid compounds can be converted to estrogens without prior metabolism to simple open chain compounds such as acetate or acetoacetic acid.

INTRODUCTION

There are two pathways involved in the formation of estrogens in the pregnant mare: (1) acetate → mevalonic acid → isopentenyl pyrophosphate → squalene → cholesterol → estrogens, estrone, 17β-estradiol and 17α-estradiol and (2) a cholesterol independent alternate pathway: acetate → mevalonic acid → isopentenyl pyrophosphate → farnesyyl pyrophosphate → ring B unsaturated estrogens, equilin* and equilenin [1–6]. Dorfman[7] had suggested that ring B unsaturated estrogens equilin and equilenin may be derived from the condensation of an ethyl toluene residue and C_{10} isoprenoid unit. Other theoretical possibilities of steroidogenesis via pathways not involving cholesterol have also been suggested [8–11]. One could speculate that ring A of these ring B unsaturated estrogens was preformed and that isopentenyl pyrophosphate or similar intermediate could then couple with it and complete the steroid structure. The coupling of tyrosine or phenylalanine metabolite p-hydroxy benzoic acid to isopentenyl pyrophosphate has been reported in the formation of umbiquinones [12]. In order to rule out the possibility that a preformed benzenoid (aromatic) compound might act as a specific precursor for equilin and equilenin, we have studied the precursor role of [U-^{14}C]-tyrosine in the formation of steroids in the pregnant mare by injecting a mixture of [^{14}C]-tyrosine and [7-^{3}H]-dehydroepiandrosterone into the fetus. The [^{3}H]-dehydroepiandrosterone serves as a control on the integrity and viability of the animal preparation since it has been previously shown that the pregnant mare efficiently converts this precursor to estrone [1–5].

EXPERIMENTAL PROCEDURES

Materials

The [U-^{14}C]-tyrosine (380 mCi/mmol, hydrochloride) and [7-^{3}H]-dehydroepiandrosterone (10.4 Ci/mmol) were obtained from New England Nuclear Corporation, Boston, U.S.A. The purity of [^{3}H]-dehydroepiandrosterone was over 95% when checked by the isotope dilution technique, the [^{14}C]-tyrosine was used as such. The injection mixture was prepared by dissolving 4.4×10^{9} d.p.m. of [^{14}C]-tyrosine and 32.0×10^{6} d.p.m. of [^{3}H]-dehydroepiandrosterone in 5 ml of a mixture (1:1) of sterile ethanol and normal saline.

The mare

The experimental subject weighed 404 kg and was approx. 300 days pregnant at the time of the operation. A laparotomy was performed and the isotope mixture (5 ml) was slowly injected into the muscles of a rear hind leg of the foal [3]. Maternal urine was collected for 5 days as described previously [5]. The mare foaled a normal 32 kg colt 5 weeks after the operation.

Methods. The methods used for the hydrolysis of urinary conjugates, the scheme of extraction, chromatography on thin layer silica gel, paper, sephadex LH-20 and Celite, detection of steroids, measurement of radioactivity and means of identification of metabolites have been described previously [3, 5, 6]. In

* The following trivial names have been used: Equilin = 3-hydroxy-1,3,5(10),7-estratetraen-17-one; Equilenin = 3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one; 17α-estradiol = 1,3,5(10)-estratriene-3,17α-diol.

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brief, the procedure consisted of hydrolysis of urinary conjugates, separation into neutral and phenolic fractions, purification of the extracts by chromatography, and whenever possible purification of metabolites by crystallization and identification by infrared spectroscopy and melting points. Radioactive metabolites were either crystallized to constant specific activity directly, or after addition of carrier steroid, and this was followed by the formation of a derivative and recrystallization to constant specific activity. Radiochemical purity was established for each of the metabolites isolated.

RESULTS

Of the dose of isotopes injected, a total of 2% [^{14}C] was recovered from the maternal urine in 5 days. The amount of [^{3}H] excreted in the urine could not be determined accurately due to the very high [^{14}C]/[^{3}H] ratio. The crude phenolic fraction (21.7 g) contained 6.8×10^6 d.p.m. of [^{14}C] (0.156% of injected dose) and 14.5×10^6 d.p.m. of [^{3}H] (45% of injected dose) and the neutral fraction (9.6 g) contained 1.4×10^6 d.p.m. [^{14}C] (0.032%) and 2.5×10^6 d.p.m. [^{3}H] (7.8%). Each fraction was processed essentially as described previously [1–3].

Phenolic fraction

The phenolic fraction was purified on a 1200 g Celite partition column (length: 120 cm, width: 7 cm) using system Benzene–Skellysolve C (1:1)–1 N NaOH [5]. The radioactive materials eluted from the column in various fractions were pooled (Pools I–XIII) as shown in Fig. 1. From Pool IV and V, estrone and equilin were isolated, identified and their radiochemical purity was established as follows:

Estrone. The residue from Pool IV (Fig. 1) weighed 298.7 mg and contained 4.9×10^6 d.p.m. of [^{3}H] and 1.3×10^5 d.p.m. of [^{14}C]. Radiochemical purity of this impure material was established by crystallization to constant specific activity both before and after the formation of the acetate derivative as shown in Table 1. The crystalline material obtained was identified as estrone [5]. The minimum percent conversion of [^{3}H]-dehydroepiandrosterone and [^{14}C]-tyrosine to estrone was 15 and 0.0003% respectively.

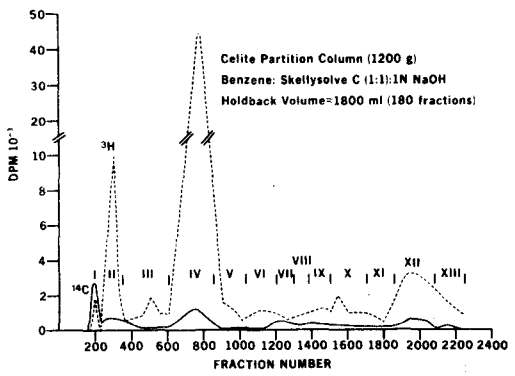

Fig. 1. Purification of the phenolic fraction by Celite partition column chromatography.

Table 1. Proof of radiochemical purity of phenolic steroids isolated after injection of [^{14}C]-tyrosine and [^{3}H]-dehydroepiandrosterone into a horse fetus

Crystallization	S.A. (d.p.m./mg)			
	Estrone*		Estrone acetate†	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
1	35160	780	33370	720
2	37600	810	33250	710
3	38024	820	32760	710
Calculated	16400	440	32900	710
	Equilin‡		Equilin acetate†	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
1	680	80	0	40
2	320	80	0	50
3	150	70	0	50
Calculated	2860	130	—	50

* A total of 298.7 mg of crude estrone containing 4.9×10^6 d.p.m. of [^{3}H] and 1.3×10^5 d.p.m. of [^{14}C] was isolated. The calculated value is based on these figures.

† The third crystals were acetylated and the specific activities have been corrected for changes in molecular weights.

‡ A total of 15 mg of equilin containing 1×10^5 d.p.m. of [^{3}H] and 4.4×10^3 d.p.m. of [^{14}C] was mixed with 20 mg of carrier equilin prior to crystallization. The calculated value is based on these figures.

Equilin. The residue from Pool V (Fig. 1) weighed 153 mg and contained 3.2×10^5 d.p.m. of [^{3}H] and 3.9×10^4 d.p.m. of [^{14}C] and was further purified by chromatography and identified as equilin as described previously [5]. A total of 15 mg of equilin containing 1.0×10^5 of [^{3}H] and 4.4×10^3 d.p.m. of [^{14}C] was recovered. At this stage, 20 mg of carrier equilin was added and the mixture crystallized. As can be seen in Table 1, the [^{3}H] S.A. was not constant indicating presence of impurities. However, after acetylation, the tritiated impurity was removed and the [^{14}C] S.A. remained constant. The minimum percent conversion of [^{14}C]-tyrosine to equilin was 0.00005%.

Neutral fraction

The neutral fraction weighing 9.6 g and containing 2.5×10^6 d.p.m. of [^{3}H] and 1.44×10^6 d.p.m. of [^{14}C] was chromatographed on a 600 g Celite partition column using the system: isooctane–t-butanol–methanol–water (10:2:7:1, by Vol.), as described previously [1]. The radioactive materials eluted from the column in the various fractions were pooled (I–VIII) as shown in Fig. 2. From Pools III, VI, and VIII, three metabolites were isolated, identified and their radiochemical purity was established as follows:

3β-Hydroxy-5α-pregnan-20-one. The crude residue from Pool III (763.4 mg and containing 2.4×10^4 d.p.m. of [^{3}H] and 1.8×10^5 d.p.m. of [^{14}C]), was purified and the crystals (260 mg)

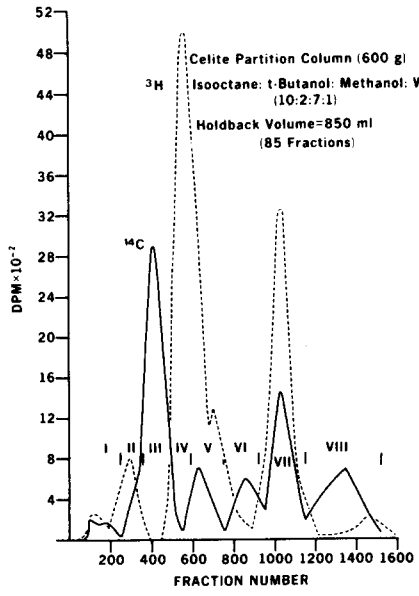

Fig. 2. Purification of the neutral fraction by Celite partition column chromatography.

obtained were identified as 3β-hydroxy-5α-pregnan-20-one [1, 3]. Radiochemical purity of this material was established by crystallization to constant specific activity both before and after the formation of the acetate as shown in Table 2. The crystals as expected, were devoid of any tritium labeled material. The minimum percent conversion of [^{14}C]-tyrosine to 3β-hydroxy-5α-pregnan-20-one was 0.004%.

5α-Pregnane-3β-20β-diol. The residue from Pool VI (600 mg and containing 5.4 × 10^4 d.p.m. of [^{3}H] and 1.0 × 10^5 d.p.m. of [^{14}C]) was purified and the crystals (190 mg) obtained were identified as 5α-pregnane-3β,20β-diol [1, 3]. Radiochemical purity of this material was established by crystallization to constant specific activity as shown in Table 2. The minimum percent conversion of [^{14}C]-tyrosine to 5α-pregnane-3β,20β-diol was 0.0023%.

5α-Pregnane-3β,20α-diol. The residue from Pool VIII (744 mg and containing 1.4 × 10^6 d.p.m. of [^{3}H] and 1.9 × 10^5 d.p.m. of [^{14}C]), was purified and the crystals (310 mg) obtained were identified as 5α-pregnane-3β,20α-diol [1, 3]. Radiochemical purity of this material was established by recrystallization to constant specific activity as shown in Table 2. The minimum percent conversion of [^{14}C]-tyrosine to 5α-pregnane-3β,20α-diol was 0.0043%.

DISCUSSION

Earlier reports [1–6, 13] have shown that the biosynthesis of the ring B unsaturated estrogens in the pregnant mare does not follow the classical pathway of steroidogenesis. In order to delineate this alternate

Table 2. Proof of radiochemical purity of neutral steroids isolated after the injection of [^{14}C]-tyrosine and [^{3}H]-dehydroepiandrosterone into a horse fetus

Crystallization	S.A. (d.p.m./mg)			
	3β-Hydroxy-5α-Pregnan-20-one*		3β-Acetoxy-5α-pregnan-20-one†	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
1	0	690	0	600
2	0	690	0	590
3	0	680	0	590
Calculated	30	230	0	600
	5α-Pregnane-3β,20β-diol‡		5α-Pregnane-3β,20β-diacetate†	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
1	0	570	0	450
2	0	550	0	450
3	0	520	0	460
Calculated	90	170	0	430
	5α-Pregnane-3β,20α-diol§		5α-Pregnane-3β,20α-diacetate†	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
1	30	580	0	520
2	0	640	0	520
3	0	610	0	500
Calculated	190	260	0	480

* A total 763 mg of crude 3β-hydroxy-5α-pregnan-20-one containing 1.8 × 10^5 d.p.m. of [^{14}C] and 2.4 × 10^4 d.p.m. of [^{3}H] was isolated. The calculated values are based on these figures.

† The third crystals were acetylated and the calculated value has been corrected for changes in molecular weight.

‡ A total of 600 mg of crude 5α-pregnane-3β,20β-diol containing 1.0 × 10^5 d.p.m. of [^{14}C] and 5.4 × 10^4 d.p.m. of [^{3}H] was isolated. The calculated values are based on these figures.

§ A total of 744 mg of crude 5α-pregnane-3β,20α-diol containing 1.9 × 10^5 d.p.m. of [^{14}C] and 1.4 × 10^5 d.p.m. of [^{3}H] was isolated. The calculated values are based on these figures.

Table 3. Endogenous specific activity and weight of steroids isolated from maternal urine following the injection of [^{14}C]-tyrosine and [^{3}H]-dehydroepiandrosterone into a horse fetus

Steroid isolated	S.A. (d.p.m./μmol)		Weight (mg)
	[^{3}H]	[^{14}C]	
Estrone	10270	220	130
Equilin	0	45	15
3β-Hydroxy-5α-Pregnan-20-one	0	220	260
5α-Pregnane-3β,20β-diol	0	170	190
5α-Pregnane-3β,20α-diol	0	200	310

pathway, we have studied the precursor role of [U-^{14}C]-tyrosine in their formation. The results obtained are summarized in Table 3. Thus, following the injection of [7-^{3}H]-dehydroepiandrosterone and [^{14}C]-tyrosine into the horse fetus, estrone, equilin, 3β-hydroxy-5α-pregnan-20-one, 5α-pregnane-3β,20β-diol and 5α-pregnane-3β,20α-diol were isolated and identified from the maternal urine (Table 3). As expected from previous experiments [^{3}H] was found only in estrone [1–5]. However, all five of the steroids isolated contained carbon-14 indicating that an aromatic amino acid-like tyrosine was utilized as precursor for steroids. This is the first report of *in vivo* steroid formation from an aromatic amino acid, though *in vitro* formation of cholesterol from glycine and serine by fungi, yeast and rat liver have been reported previously [14].

Since the [^{14}C]-tyrosine used in our study was uniformly labeled it would seem that tyrosine is first metabolized to simple open chain compounds such as acetoacetic acid prior to being incorporated into steroids. The conversion of acetoacetic acid to cholesterol has been reported previously [15–17]. Thus, it is unlikely that a preformed benzenoid compound condenses with an isoprenoid unit(s) to form ring B unsaturated estrogens [7].

The [^{14}C] S.A. of estrone (220 d.p.m./μmol) and the pregnane derivatives (170–220 d.p.m./μmol) are quite similar indicating that both the classical estrogens and neutral steroids were probably formed via pathways utilizing the same precursors and intermediates. In contrast to these findings, the specific activity of equilin is approx. five times lower than that of estrone. Similar differences were observed in all of our previous studies.

Whether aromatic amino acids participate in the biosynthesis of steroids in other species remains to be established. The physiological significance of this finding is not yet clear. However, it may be of importance to investigate the role of aromatic amino acids derived from dietary protein in the endogenous formation of cholesterol.

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REFERENCES

1. Bhavnani B. R. and Short R. V.: Formation of steroids by the pregnant mare. III. Metabolism of ^{14}C-squalene and ^{3}H-dehydroisoandrosterone injected into the fetal circulation. *Endocrinology* **92** (1973) 657–666.
2. Bhavnani B. R. and Short R. V.: Formation of steroids by the pregnant mare. IV. Metabolism of ^{14}C-mevalonic acid and ^{3}H-dehydroisoandrosterone injected into the fetal circulation. *Endocrinology* **92** (1973) 1397–1404.
3. Bhavnani B. R., Martin L. J. and Baker R. D.: Formation of steroids by the pregnant mare. V. Metabolism of ^{14}C-isopentenyl pyrophosphate and [^{3}H]-dehydroisoandrosterone injected into the fetus. *Endocrinology* **96** (1975) 1009–1017.
4. Bhavnani B. R. and Woolever C. A.: Formation of steroids by the pregnant mare. VI. Metabolism of ^{14}C-farnesyl pyrophosphate and ^{3}H-dehydroepiandrosterone injected into the fetus. *Endocrinology* **103** (1978) 2291–2298.
5. Bhavnani B. R., Short R. V. and Solomon S.: Formation of estrogens by the pregnant mare. I. Metabolism of 7-^{3}H-dehydroisoandrosterone and 4-^{14}C-androstenedione injected into the umbilical vein. *Endocrinology* **85** (1969) 1172–1179.
6. Bhavnani B. R., Short R. V. and Solomon S.: Formation of estrogens by the pregnant mare. II. Metabolism of ^{14}C-acetate and ^{3}H-cholesterol injected into the fetal circulation. *Endocrinology* **89** (1971) 1152–1157.
7. Dorfman R. I.: Metabolism of androgens, estrogens and corticoids. *Am. J. Med.* **21** (1956) 679–687.
8. Hechter O.: Conversion of cholesterol to steroid hormones. In *Cholesterol* (Edited by R. P. Cook). Academic Press, New York (1958) pp. 309–347.
9. Tait A. D.: The conversion of 23,24-dinor-chol-5-en-3β-ol to steroid hormones. *Biochem. J.* **128** (1972) 467–470.
10. Tait A. D.: The biosynthesis of 23,24-dinor-5-cholene-3β,20-diol and 23,24-dinor-5-cholene-3β,21-diol. *Steroids* **22** (1973) 239–243.
11. Tait A. D.: The conversion of 23,24-dinor-5-cholene-3β-ol to cortisol by the canine adrenal. *Steroids* **22** (1973) 609–613.
12. Rudney H.: The biosynthesis of terpenoid quinones. In *Natural Substances formed Biologically from Mevalonic acid* (Edited by T. W. Goodwin). Academic Press, New York (1970) pp. 89–103.
13. Savard K.: The estrogens of the pregnant mare. *Endocrinology* **68** (1961) 411–416.
14. Bose A. K. and Hungund B. L.: The role of glycine in the biosynthesis of steroids. *Experientia* **29** (1973) 939–940.
15. Curan G. L.: Utilization of acetoacetic acid in cholesterol synthesis by surviving rat liver. *J. biol. Chem.* **191** (1951) 775–782.
16. Brady R. O. and Gurin S.: The synthesis of radioactive cholesterol and fatty acids *in vitro*. *J. biol. Chem.* **189** (1951) 371–377.
17. Zabin I. and Bloch K.: The utilization of butyric acid for the synthesis of cholesterol and fatty acids. *J. biol. Chem.* **192** (1951) 261–266.