On the Relative Conversion of 6,7-^{3}H-Estrone to ^{3}H-15α-Hydroxyestrogens and ^{3}H-7α-Hydroxyestrogens by *Glomerella fusarioides.*

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SUMMARY

Mixtures of 6, 7-^{3}H-estrone and 16-^{14}C-estrone were fermented in media of Glomerella fusarioides. *Conversions of each form of estrone to 15α-hydroxyestrone (15α-OHE_1) and 7α-hydroxyestrone (7α-OHE_1) and in one experiment to 15α-hydroxyestradiol (15α-OHE_2) and 7α-hydroxyestradiol (7α-OHE_2) were compared. The metabolites were separated and purified by repeated column chromatography on Celite. Similar to results reported for non-isotope studies, ^{14}C-estrone was converted to more 7α-hydroxyestrogens than 15α-hydroxyestrogens. The reverse was true for the tritiated substrate. The difference is attributed to an isotope effect resulting in diminished hydroxylation of the tritiated substrate at C-7 with concomitant enhanced hydroxylation at C-15 where no isotope effect would be expected. Reduction to 17β-hydroxyestrogens also occurred, but quantitative significance for this pathway was confined to the 15α-hydroxy compound.*

INTRODUCTION.

15α-Hydroxyestrone (3, 15α-dihydroxy-1, 3, 5 (10)-estratrien-17-one; 15α-OHE_1) and 15α-hydroxyestradiol (1, 3, 5, (10)-estratriene-3, 15α-17β-triol; 15α-OHE_2) have been isolated from fetal tissues and maternal urine in human pregnancy [1,2,3] and more recently from human bile in non-pregnancy [4]. These studies pointed to their importance in the scheme of estrogen

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metabolism. No method for the synthesis of labelled 15α-OHE_1 or 15α-OHE_2 has been published.

In seeking to achieve the synthesis of ^{3}H-15α-OHE_1, two biosynthetic methods were considered. The first, a two-step procedure, produces 15α-OHE_1 in good yield, but utilizes estr-4-ene-3, 17-dione, a compound not available in a labelled form[5]. The second is a one-step synthesis from estrone by Glomerella fusarioides, but partially owing to the predominance of 7α-hydroxylation and 17β-hydroxyreductase activity, the yield is poor [6]. Nevertheless, considering the availability and low cost of 6, 7-^{3}H-estrone the latter method appeared more attractive.

We found that an isotope effect resulted in a higher conversion of 6, 7-^{3}H-estrone to ^{3}H-15α-OHE_1 relative to ^{3}H-7α-hydroxyestrone (3, 7α-dihydroxy-1, 3, 5 (10)-estratrien-17-one; 7α-OHE_1). Furthermore, the yield of 15α-OHE_2 clearly exceeded that of 7α-hydroxyestradiol (3, 7α, 17β-trihydroxy-1, 3, 5 (10)-estratriene; 7α-OHE_2). In addition to presenting a chromatographic scheme for the separation and purification of labelled 15α-OHE_1 and 7α-OHE_1; 15α-OHE_2 and 7α-OHE_2, this paper concentrates on some of the quantitative aspects of the isotope effect. The experimental design was to compare the ^{3}H/^{14}C ratio of 15α-OHE_1 with that of 7α-OHE_1 as well as 15α-OHE_2 to that of 7α-OHE_2 following incubation of the mold with a mixture of 6, 7-^{3}H-estrone and 16-^{14}C-estrone. The 16-^{14}C-labelled compound would not be subject to an isotope effect at the 7 and 15 positions, and therefore serves as a convenient frame of reference.

EXPERIMENTAL.

Materials.

6, 7-^{3}H-Estrone was purchased from the Amersham/Searle Corporation. The Company reports that at least 95 % of the ^{3}H is distributed between the 6 and 7 position but the orientation of the isotope was not determined. 16-^{14}C-Estrone was synthesized in this laboratory [7]. A mixture containing 8.0 μCi of ^{3}H and 1.95 μCi of ^{14}C was purified by sequential column chromatography on Celite in systems 1 and 2 (Table 1). Estrone was eluted in fractions 19-22 and 17-20, respectively. The purified mixture was divided into two parts : one was diluted with 2 mg of estrone and the other with 20 mg of estrone.

Column Chromatography.

Acid washed Celite 545 (Johns-Manville, Manville, N.J. 08835) was washed twice with acetone and air-dried before use. Thirty-six g of Celite were mixed with 18 ml of stationary phase and packed into a 2.0 × 45 cm

TABLE 1. Celite column chromatography systems.

System 1 [a]	
Stationary phase	13 ml ethylene glycol in 26 g of Celite above 3 ml H_2O in 10 g of Celite
Mobile phase	700 ml isooctane
Gradient solvent	475 ml ethyl acetate (gradient started at Tube # 4)
System 2[b]	
Stationary phase	18 ml lower phase obtained from isooctane : ethyl acetate : methanol : water (20 : 10 : 12 : 8)
Mobile phase	700 ml upper phase obtained from isooctane : methanol : water (7 : 6 : 5)
Gradient solvent	300 ml ethyl acetate
System 3[b]	
Stationary phase	18 ml upper phase obtained from chloroform : hexane : methanol : water : 1, 2-dichloroethane (8 : 9 : 7 : 3 : 4)
Mobile phase	840 ml lower phase obtained from chloroform : hexane : methanol : water (480 : 360 : 280 : 120)
Gradient solvent	1, 2-dichloroethane (gradient started at Tube # 5)

[a] 10 ml cuts were taken. At tube 90 the mobile phase was replaced with 200 ml ethyl acetate.

[b] 5 ml cuts were taken.

column according to the directions of Siiteri [8]. The sample was dissolved in 1.5 ml of stationary phase, mixed with 3 g of Celite and packed on top. A gradient elution technique using the apparatus described by Engel *et al.* was employed [9]. Unless indicated otherwise, the gradient was started as soon as the first drop of solvent appeared at the bottom of the column and 10 ml cuts were taken.

Fermentation.

Culture media of *Glomerella fusarioides* (ATCC 9552) were prepared as described by Laskin *et al.* [6]. Solutions of 2 mg and 20 mg of the double-labelled estrone in N, N-dimethylformamide were added respectively to two 1 liter flasks each containing 200 ml of media. Following incubation for 2 days, the contents of the flask were filtered and extracted with chloroform.

Purification of 15α-*Hydroxyestrone and* 7α-*Hydroxyestrone.*

The chloroform extract was dried and chromatographed in system 1. The distribution of radioactivity in the 2 mg incubation is shown in Figure 1. Fractions 54-58 (15α-OHE_1) and 59-63 (7α-OHE_1) were chromatographed separately in the same system twice more. In order to verify radiochemical

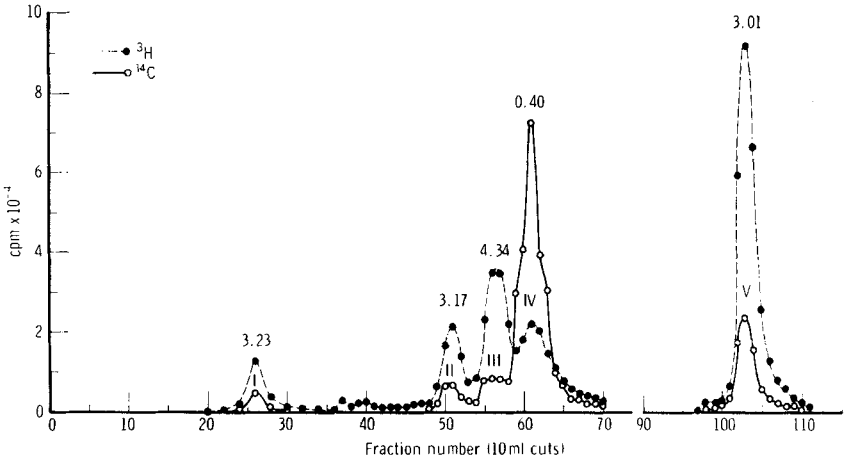

FIG. 1. Distribution of radioactivities in system 1 following fermentation of 2 mg of a mixture of 6,7-^{3}H-estrone and 16-^{14}C-estrone ($^3H/^{14}C = 2.67$) with *Glomerella fusarioides*. Zone I corresponds to estrone; zone III to 15α-OHE_1; zone IV to 7α-OHE_1 and zone V to 7α-OHE_2 + 15α-OHE_2. Values above peaks refer to the $^3H/^{14}C$ ratios of the entire zones.

purity, the 3-benzyl ethers were prepared (10) and chromatographed in system 1. These derivatives of 15α-OHE_1 and 7α-OHE_1 were eluted in fractions 34-38 and 38-40, respectively. The peaks were sharp and separable provided 5 ml cuts were taken. Finally, the 3-benzyl ether of 7α-OHE_1 was reduced to 7α-OHE_2-3-benzyl ether with $NaBH_4$ [11]. The product was eluted in fractions 31-33 in system 1. At each stage of purification the samples were counted in a Model 3375 Packard TriCarb Scintillation Counter and the $^3H/^{14}C$ ratios were calculated.

Separation and Purification of 15α-Hydroxyestradiol and 7α-Hydroxyestradiol.

Zone V (Fig. 1) was pooled and chromatographed in system 3. 7α-OHE_2 was eluted in fractions 27-34 and 15α-OHE_2 was eluted in fractions 36-44. Following further chromatography in the same system, the 3-benzyl ether of each compound was prepared and chromatographed in system 1. The 3-benzyl ether of 7α-OHE_2 appeared in fractions 31-33 and that of 15α-OHE_2, in fractions 32-35. The $^3H/^{14}C$ ratio was determined at each stage of purification.

RESULTS AND DISCUSSION.

Following fermentation of double-labelled estrone with *Glomerella fusarioides*, about 30-40 % of the radioactivity was chloroform extractable.

TABLE 2. $^{3}H/^{14}C$ Ratios in 15α-hydroxyestrone (15α-OHE_1), 7α-hydroxyestrone (7α-OHE_1), 15α-hydroxyestradiol (15α-OHE_2) and 7α-hydroxyestradiol (7α-OHE_2) at various stages of purification and derivative formation after fermentation of double-labelled estrone (E_1) with *Glomerella fusarioides*.

	Experiment 1 2 mg E_1				Experiment 2 20 mg E_1	
	15α-OHE_1	7α-OHE_1	15α-OHE_2	7α-OHE_2	15α-OHE_1	7α-OHE_1
Compound	$^{3}H/^{14}C$				$^{3}H/^{14}C$	
Parent	4.54	0.40	4.16	0.50	4.08	0.61
	4.40	0.37	4.25	0.55	4.41	0.48
				0.36	4.39	0.38
Derivatives						
3-benzyl ether	4.41[a]	0.32[a]	4.11	0.32	4.59[a]	0.35[a]
Reduced 3-benzyl ether		0.33				0.35

Values are those obtained following sequential chromatography in the systems indicated in the text. $^{3}H/^{14}C$ ratio of starting compound was 2.67.

[a] Chromatographed in system 1, modified in that 5 ml cuts were taken and the gradient was started in tube 1.

Chromatography in system 1 resulted in 5 defined zones of radioactivity (Fig. 1). Zone 1 represents unreacted estrone; zone III, 15α-OHE_1; zone IV, 7α-OHE_1, and zone V, a mixture of 15α-OHE_2 and 7α-OHE_2. 15α-OHE_2 and 7α-OHE_2 are products of fermentation [6], but they are not separable in the system employed. Zone II was not investigated further.

The $^{3}H/^{14}C$ ratios of 15α-OHE_1 and 7α-OHE_1 at each state of purification are shown in Table 2. It can be seen that constant ratios were achieved for 15α-OHE_1 at an early stage, the second chromatography in system 1. Parenthetically, ^{3}H-15α-OHE_1 has been synthesized by this method in our laboratory several times. After the second elution from Celite, reverse isotope dilution analysis invariably indicated a pure preparation.

The interesting result of this is the high $^{3}H/^{14}C$ ratio in 15α-OHE_1 relative to that in 7α-OHE_1 and the substrate. In non-isotope fermentations, 7α-OHE_1 is produced in about 4-6 % yield, some 3-4 times greater than 15α-OHE_1 [6]. The estimated yields in our studies are shown in Table 3. Although the yields appear to be influenced by the substrate concentration, it can be seen that the yield of ^{14}C-7α-OHE_1 clearly exceeded that of ^{14}C-15α-OHE_1. On the other

TABLE 3. Yields of labelled 15α-hydroxyestrone (15α-OHE_1), 7α-hydroxyestrone (7α-OHE_1), 15α-hydroxyestradiol (15α-OHE_2) and 7α-hydroxyestradiol (7α-OHE_2) obtained on fermentation of double-labelled estrone with *Glomerella fusarioides*.

		% Conversion to			
		15α-OHE_1	7α-OHE_1	15α-OHE_2	7α-OHE_2
Experiment 1					
2 mg estrone	^3H	4.5	2.5[a]	7.0	< 0.1[a]
	^{14}C	2.7	20	4.0	< 0.5
Experiment 2					
20 mg estrone	^3H	2	0.4	—	—
	^{14}C	1.5	3.3	—	—

[a] See footnote.

hand, the yield of ^3H-15α-OHE_1 exceeded that of ^3H-7α-OHE_1[a]. A plausible explanation for this difference is that an isotope effect resulted in diminished hydroxylation by *Glomerella fusarioides* of the tritiated substrate at C-7 with a concomitant enhancement of hydroxylation at C-15 where no isotope effect would be expected. Assuming that approximately 50 % of the ^3H in the estrone is 7α-oriented, the isotope effect estimated from the relative conversions to ^3H-7α-OHE_1 and ^{14}C-7α-OHE_1 is about 400 %. By analogy with other microbial hydroxylations the formation of 7α-and 15α-hydroxylated estrogens would proceed by the direct replacement of the α-oriented H by OH [12].

The mixture in zone V of Figure 1 was separated into 15α-OHE_2 and 7α-OHE_2, the $^3H/^{14}C$ ratios of which were similar to those of the corresponding 17-keto compounds (Table 2). Thus, 17β-hydroxy reductase activity appears to be independent of the isotope effect. However, this enzyme activity in *Glomerella fusarioides* is strongly influenced by the position of the hydroxy group. The yield of 15α-OHE_2 exceeded that of 15α-OHE_1 (whereas 7α-OHE_2 was barely detectable (Table 3). However, in this regard the incubation time is important since reduction at C-17 increases as the incubation time is extended [6].

The isotope effect indirectly renders the one-step synthesis of ^3H-15α-OHE_1 and ^3H-15α-OHE_2 from 6, 7-^3H-estrone by *Glomerella fusarioides* attractive. It is conceivable that other microorganisms containing 2 or more hydroxylating

[a] Most likely the isotope yield is not an accurate index of the conversion to ^3H-7α-OHE_1. The 6,7-^3H-estrone prepared by the catalytic tritiation of the corresponding 6-dehydro compound probably contains the bulk of the ^3H evenly distributed between the 6α and 7α positions. Thus, the true conversion to ^3H-7α-OHE_1 may be as much as twice the isotope yield.

enzyme systems may be exploited in a similar manner by the selective positioning of ^{3}H. On the other hand, it is cautioned that *in vivo* and *in vitro* metabolism studies involving hydroxylation at ^{3}H-labelled sites in the steroid nucleus may yield biased data.

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