SYNTHESIS OF 16α-^{3}H ANDROGEN AND ESTROGEN SUBSTRATES FOR 16α-HYDROXYLASE

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ABSTRACT

The synthesis of 16α-^{3}H androgens and estrogens is described. 1-(^{3}H)-Acetic acid in the presence of zinc dust reacts with 16α-bromo-17-ketosteroids to produce 16α-^{3}H-17-ketosteroids. This chemical reaction was used to prepare 16α-^{3}H-dehydroepiandrosterone (I) and 16α-^{3}H-estrone acetate (XI) from 16α-bromo-dehydroepiandrosterone (X) and from 16α-bromo-estrone acetate (XII), respectively. Using appropriate microbiological techniques, it was possible to convert these radiolabelled substrates into 16α-^{3}H-androstenedione (II) and 16α-^{3}H-estradiol-17β (VII). 16α-^{3}H-Estrone (VI) was obtained by the chemical hydrolysis of 16α-^{3}H-estrone acetate. The label distribution as determined by microbiological 16α-hydroxylations indicated a specific labelling of 77% for androgens and 65% for estrogens in the 16α position. These substrates can be used for measuring the 16α hydroxylase activity, an important step in the biosynthesis of estriol (VIII) and estetrol (IX).

INTRODUCTION

Several papers (1,2,3) have clearly reported that the daily production of estriol and estetrol during pregnancy can be regarded as a good index of fetal well-being, because 15α and 16α hydroxylations take place primarily in the fetal liver. A study of the biochemical properties of these hydroxylases may represent a major breakthrough in the understanding of the biosynthesis of these two estrogens.

The conventional method for estimating enzymatic activities requires the incubation of ^{14}C or ^{3}H labelled substrates, elaborate laboratory manipulations (extractions, purifications and identification of all metabolites), as well as final liquid scintillation counting. As the steroid substrates are transformed into large numbers of metabolites having closely related physico-chemical properties, this method is cumbersome and time-consuming.

It was necessary to develop a single, accurate, sensitive and specific method for measuring these enzymatic activities more rapidly. The evaluation of the ^{3}H-water released during the incubation of a stereospecifically labelled substrate provides a unique assay procedure. In our laboratory, such isotopic techniques have already been successfully used in evaluating various enzymatic activities (4,5,6,7,8).

This report describes both the synthesis and purification of 16α-^{3}H precursors of the formation of estriol and estetrol *in vivo*, and the labelling distribution established by various chemical, physical and microbiological methods. 16α-^{3}H-Dehydroepiandrosterone, 16α-^{3}H-androstenedione, 16α-^{3}H-estrone and 16α-^{3}H-estradiol were prepared. Their synthesis necessitated different steps involving successive chemical and microbiological reactions. The overall procedure included the production of a 16-bromo derivative followed by a reduction with native tritium and a subsequent microbiological reaction or a chemical hydrolysis, leading to the preparation of the final tritiated compound. Figure 1 represents the synthetic pathways for the four compounds.

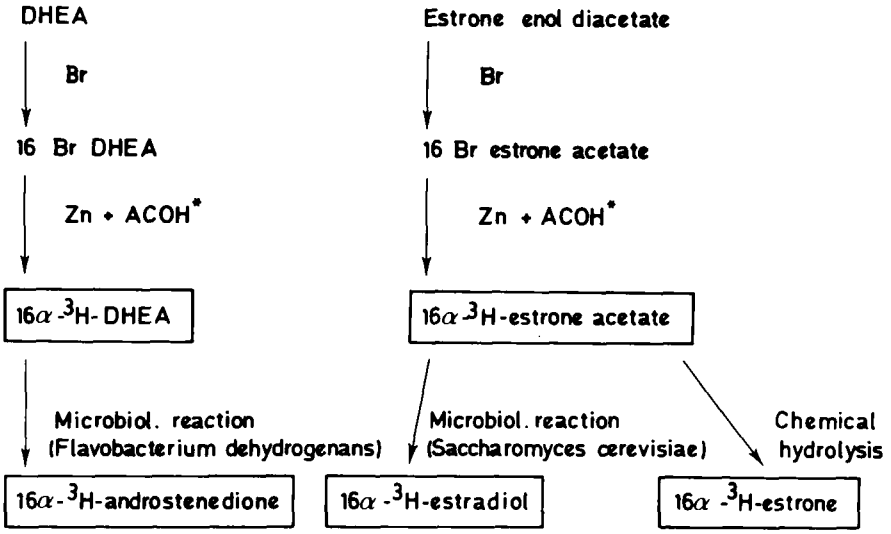

FIGURE 1

EXPERIMENTAL

A. Materials

1. Chemicals

All of the chemicals were of analytical grade quality (Merck).

- The solvents for the tritiation and deuteration reactions were redistilled before use. Ethyl ether was dried over sodium pellets and distilled on calcium hydride. Acetic anhydride was distilled over anhydrous calcium chloride.
- ^{3}H-Water (5 Ci/ml) was from Amersham (U.K.), ^{14}C androstenedione and ^{2}H-acetic acid were from I.R.E. (Fleurus, Belgium). 1-^{3}H-Acetic acid was prepared from 1 Ci of ^{3}H-water mixed with 1.1 ml of acetic anhydride.
- Methoxyamine hydrochloride, trimethylchlorosilane (TMCS), bistrimethylsilylacetamide and dry pyridine were used to prepare the MO-TMS derivatives for the GLC separations. These compounds were from Macherey-Nagel (Düren, F.R.G.).
- Scintillation product (Picofluor) was from Packard Instruments (Groningen, The Netherlands).
- Estrone enol diacetate (XIII) was from Steraloids Inc.

2. Biological elements

Flavobacterium dehydrogenans, Streptomyces halstedii and Streptomyces roseochromogenus strains were from the American Type Culture Collection (Rockville, Maryland). Saccharomyces cerevisiae was from the Centraalbureau voor Schimmelculturen (Baarn, The Netherlands).

B. Instruments

Pre-coated thin layer chromatographic plates, Siligur-25 UV_{254} were from Macherey-Nagel (Düren, F.R.G.). Gas chromatographic analyses were performed on a Packard instrument, model 419, equipped with a flame ionization detector. High performance liquid chromatographic separations were performed on a Waters instrument, model 440. The ^{13}C nuclear magnetic resonance spectra were obtained on a Bruker HFX-90 instrument, operating at 22.63 MHz, field locked on deuterium, with broad band proton decoupling. 7,000-20,000 transients were required to obtain the spectra, in the Fourier transform mode, for the steroids as saturated solutions in benzene-d6. Mass spectra were recorded on a LKB 2091 gas chromatograph-mass spectrometer instrument (Bromma, Sweden). A Packard Tricarb 3375 scintillation counter was used for measuring radioactivity.

C. Synthesis and purification of the 16α-^{3}H substrates

1. 16α-Bromo-3β-hydroxy-5-androsten-17-one

This bromo compound was prepared from DHEA (200 mg) according to the technique described by Glazier (9) and Numazawa and Osawa (17). The reaction time was quite long (72 hours) and the yield was 55%. The bromo derivative was separated from DHEA by a liquid chromatographic method using a Lobar silica gel 60 column eluted with a benzene/ethyl acetate mixture (200 ml) (9/1; v/v); mp 176-177°.

2. 16α-^{3}H-3β-Hydroxy-5-androsten-17-one

16α-Bromo-3β-hydroxy-5-androsten-17-one (100 mg) dissolved in 20 ml dried ether was added to 5 ml of 1 Ci ^{3}H-acetic acid. Zn dust (500 mg) divided into four fractions was slowly introduced by continuous stirring into the reaction vial and the hydrogenation was performed during the following 4 to 5 hours.

Water (10 ml) was added and the resulting mixture was extracted with ethyl ether (50 ml). The upper organic layers were washed with water, dried over anhydrous sodium sulfate and evaporated to dryness. 16α-^{3}H-DHEA (60 mg) dissolved in a suitable quantity of chloroform was purified by liquid chromatography on a Lobar silica gel 60 column using a chloroform/methanol mixture (200 ml) (98/2; v/v) as eluent.

3. 16α-^{3}H-Androstenedione

16α-^{3}H-Dehydroepiandrosterone was converted into 16α-^{3}H-androstenedione by Flavobacterium dehydrogenans (14). The culture medium was made of 10 g of a Difco yeast extract, 4.5 g of Na_2HPO_4 and 3.4 g of NaH_2PO_4 per liter of distilled water. The pH was brought to 6.2 and the medium was sterilized by autoclaving for 40 min at 120°C. After inoculation, the culture was incubated for 48 hours at 31°C with continuous shaking and illumination. Five ml of this culture preparation was transferred to 50 ml of a fresh medium containing 2.5 g of yeast extract per liter. Again, this solution was incubated for the next 36 hours under the same conditions as described above. 16α-^{3}H-DHEA (10 mg) dissolved in a minimal volume of acetone was suspended in 50 ml of culture medium introduced into a 250 ml conical flask. After incubation, the medium was extracted twice with 100 ml of dichloromethane, with 100 ml of ether, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in a minimal quantity of benzene and chromatographed on a Lobar silica gel 60 column eluted with a benzene/acetone mixture (150 ml) (98/2; v/v). 16α-^{3}H-Androstenedione (6 mg) was isolated. This compound presented a single peak in GLC and its retention time was identical to that of authentic reference androstenedione.

4. 16α-Bromo-3-hydroxy-1,3,5(10)-estratrien-17-one 3-acetate

This compound was obtained by bromination of estrone enol diacetate (300 mg) according to the method of Johnson and Johns (11). The final chloroformic extract was also purified on a Lobar silica gel 60 column eluted with a benzene/ethyl acetate mixture (400 ml) (9/1; v/v). The yield was 60%; mp 168-169°.

5. 16α-^{3}H-Estradiol

16α-^{3}H-Estrone acetate was converted into 16α-^{3}H-estradiol by Saccharomyces cerevisiae (19). The culture medium contained yeast extract (10 g/l), glucose (60 g/l), Na_2HPO_4 (4.5 g/l), and NaH_2PO_4 (3.4 g/l). The pH was adjusted to 7 and the medium was sterilized by autoclaving. After inoculation, the culture was incubated for 1 day on a rotary shaker at 30°C. A 10 mg aliquot of 16α-^{3}H-estrone acetate dissolved in a few drops of acetone was suspended in 50 ml of culture medium introduced into a 250 ml conical flask and stirred for 3 days under the optimal conditions described above. The medium was extracted with 100 ml of ether and 100 ml of ethyl acetate. The combined organic layers were washed with water, dried over anhydrous sodium sulfate and evaporated to dryness. The residue, dissolved in a few drops of benzene, was purified by liquid chromatography on a Lobar silica gel 60 column eluted with a benzene/ethyl acetate mixture (150 ml) (8/2; v/v). Five mg of 16α-^{3}H-estradiol was isolated. This compound presented the same chromatographic characteristics (TLC-GLC) as authentic reference estradiol.

6. 16α-^{3}H-Estrone

This compound was obtained by the chemical hydrolysis of 16α-^{3}H-estrone acetate. 16α-^{3}H-Estrone acetate (10 mg) was solvolyzed for 6 hours at room temperature in 10 ml of a 0.1 N methanolic hydrochloric acid solution. Distilled water (10 ml) was then added to the methanolic solution and the medium was extracted with 100 ml of chloroform. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was easily purified on a Lobar column eluted with a cyclohexane/ethyl acetate mixture (200 ml) (75/25; v/v). Five mg of 16α-^{3}H-estrone was isolated.

D. Control of the chemical purity of the substrates

The chemical purity of the various substrates (16α-^{3}H-DHEA, 16α-^{3}H-androstenedione, 16α-^{3}H-estrone and 16α-^{3}H-estradiol) was controlled by TLC, HPLC and high resolution GLC after derivatization of the polar functions.

TLC was performed on a silica gel plate with a fluorescent indicator, using a mixture of benzene/chloroform/methanol (3/7/1; v/v) for the elution of the 16α-^{3}H androgens, and a mixture of cyclohexane/ethyl acetate (75/25; v/v) for the elution of the 16α-^{3}H estrogens.

HPLC analyses were carried out on a μBondapack C_{18} column (3.9 mm x 30 cm) (Waters Associates, Belgium), using an isocratic methanol/water (7/3; v/v) elution mixture at a 1 ml/min flow rate. The compounds were detected at 254 and 280 nm.

High resolution gas-liquid chromatographic separations were performed after transformation of the molecules into MO-TMS (methoxime-trimethylsilyl ethers) derivatives prepared according to Horning's method (22). The separations were carried out with a glass capillary column (50 m long; 0.25 mm internal diameter) coated with SE-30 and heated at 240°C. Helium was used as a carrier gas at a pressure of 0.6 bar. A solid injector device was used. The flame ionization detector was set at 260°C.

E. Control of the specificity of the labelling

1. Chemical methods

16α-^{3}H-Estrone (or 16α-^{3}H-androstenedione) (10 mg) was dissolved in 20 ml methanolic solution of potassium hydroxide (2 N) and stirred for 3 hours at room temperature. After neutralization by aqueous hydrochloric acid, the estrone (or androstenedione) was extracted twice by two volumes of chloroform. The radioactivity released in the aqueous solution was then counted.

2. Physical methods

^{13}C NMR spectra and mass spectra of the deuterated and corresponding unlabelled compound were compared.

3. Microbiological methods

a. *16α-^{3}H-Androstenedione*

The Streptomyces roseochromogenus culture medium, adjusted to pH 7.2, contained yeast extract (0.3 g/l), beef extract (0.3 g/l), Bacto tryptone (0.7 g/l), $FeSO_4$ (3 mg/l) and glucose (3 g/l). The microorganisms were cultivated for 1 day at 28°C under continuous shaking. 16α-^{3}H-Androstenedione and ^{14}C androstenedione (0.5 mg) dissolved in 500 µl acetone were added to the culture medium and incubated for 2 days. The combined ether and ethyl acetate extracts were washed, dried and evaporated to dryness. The residue dissolved in a minimal quantity of chloroform was chromatographed on a silica gel plate with benzene/$CHCl_3$/methanol elution mixture (3/7/1). The spots corresponding to androstenedione and 16α hydroxylated androstenedione, respectively, were scraped off and counted by liquid scintillation. The proportion of tritium atoms present in the 16α position was calculated as follows (radioactivities expressed in dpm):

$$\frac{^3H \,/\, ^{14}C \text{ (substrate)} - {}^3H \,/\, ^{14}C \text{ (16α-OH compound)}}{^3H \,/\, ^{14}C \text{ (substrate)}}$$

b. *16α-^{3}H-Estrone*

16α-^{3}H-Estrone and ^{14}C estrone (0.5 mg) were incubated with a Streptomyces halstedii culture medium under the same experimental conditions as those described for Streptomyces roseochromogenus. The specificity of the labelling in the 16α position was determined similarly to that for androstenedione.

RESULTS AND DISCUSSION

The preparation of steroids stereospecifically tritiated on a carbon adjacent to a keto group is difficult because of the possibility of the enolization of the carbonyl function. For obvious reasons (specificity of labelling, simplicity of the purification steps, influence on the specific radioactivity), it was necessary to prepare extremely pure 16α-brominated steroids. These compounds could be quickly formed in a one-step reaction. Under the experimental conditions described by Johnson and Johns (11) and by Numazawa and Osawa (17), it was evident that only the 16α position could be brominated. This monobromination was easily confirmed by mass spectrometry (one doublet at M and M+2

molecular ions in an approximate 1/1 ratio). The purification of these brominated precursors was absolutely necessary and therefore an additional liquid chromatographic purification step was performed rather than the usual recrystallizations.

The halogenated compounds having the halogen atom adjacent to a keto group can normally be transformed into the corresponding ^{3}H-steroids by an exchange procedure with native tritium produced by the reaction of ^{3}H-acetic acid with Zn dust (12). For this purpose, it was imperative to prepare very pure ^{3}H-acetic acid with the highest activity.

The last reduction (for estradiol) and oxidation (for androstenedione) reactions using labelled compounds were carried out with microbiological methods rather than with chemical reactants (Jones's reagent, Oppenhauer oxidations, $NaBH_4$ reductions). 16α-^{3}H-Androstenedione was synthesized with a microbiologically driven reaction using a Flavobacterium dehydrogenans strain which selectively transforms Δ5-3β-hydroxy to Δ4-3-ketosteroids (14). 16α-^{3}H-Estradiol was prepared using a Saccharomyces cerevisiae strain which simultaneously transforms the 17 keto group into a 17β OH function and hydrolyzes the acetyl group in position 3 of the 16α-^{3}H-estrone acetate (19). Microbiological reactions are very specific and are conducted under mild experimental conditions (neutral pH) in order to avoid a large loss of tritium and the occurrence of side reactions.

By using the methodologies described above, it was possible to synthesize the substrates to a purity which was verified by TLC, HPLC and GLC. For all of these methods of verification, the evidence of a pure compound could be established by comparison of their chromatographic properties in various systems with those of authentic reference samples.

The specific radioactivity of these 16α-^{3}H substrates was established by using a GLC procedure for DHEA and an HPLC procedure for the other compounds. The concentrations of the prepared solutions were evaluated by comparison with a calibration curve obtained from authentic reference samples. Internal standards were testosterone (III) for the 16α-^{3}H-DHEA, progesterone (IV) for the 16α-^{3}H-androstenedione, and estriol for the 16α-^{3}H-estradiol and 16α-^{3}H-estrone, respectively. These specific radioactivities are listed in Table 1.

TABLE 1

Substrates	Specific radioactivities
16α-^{3}H-DHEA	3.37 Ci/mole
16α-^{3}H-androstenedione	2.77 Ci/mole
16α-^{3}H-estrone	2.61 Ci/mole
16α-^{3}H-estradiol	3.6 Ci/mole
16α-^{3}H-estrone acetate	5.20 Ci/mole

Despite the fact that all precautions were taken to avoid the presence of water during the tritiation, the specific radioactivities attained for DHEA and estrone acetate remained below 5.3 Ci/mole. These activities were similar to those given by Kremers *et al.* for the synthesis of 17-^{3}H-pregnenolone (V) and progesterone obtained by a similar technique (7). It seems that these specific activities were really the maximum that could be obtained by such tritiation procedures. The microbiological transformation of DHEA into androstenedione took place with a minimal loss of radioactivity (18%), when compared to a conventional chemical reaction usually occurring under more rigorous conditions. The hydrochloric acid hydrolysis of estrone acetate into estrone took place with a 50% loss of radioactivity.

Control of the radiochemical purity of the substrates was per-

formed by the use of chemical, physical and microbiological methods.

A. Chemical methods

A 2 N methanolic potassium hydroxide solution produces the enolization of the carbonyl functions with an exchange of the neighboring protons. The amount of tritium released in the medium corresponds to the amount of tritium located on the carbons adjoining the keto group. 16α-^{3}H-Androstenedione and 16α-^{3}H-estrone (prepared from the same precursors as for the synthesis of 16α-^{3}H-DHEA and 16α-^{3}H-estradiol, respectively), completely released their radioactivity after this reaction. These results ascertained that all of the radioactivity was situated on a carbon adjacent to the keto group (in the 16 position).

B. Physical methods

Two physical techniques were used to confirm the labelling position. The analyses were carried out with deuterated samples prepared under exactly the same conditions as those described for the radioactive materials. Bromo ketones can be reduced to halogen-free ketones by treatment with Zn in ^{2}H-acetic acid (12). This mode of deuteration offered a means for introducing only one deuterium atom next to the carbonyl group.

1. Mass spectrometric analysis

Figures 2 and 3 represent the mass spectra obtained for 16α-^{2}H-DHEA (the precursor of androstenedione) and 16α-^{2}H-estrone acetate (the precursor of estradiol and estrone), respectively. The monodeuteration of DHEA was evidenced by the appearance of a very intense molecular peak

at m/e 289, corresponding to a molecular weight that was one mass unit higher than the unlabelled DHEA. The fragmentation pattern indicated that the deuterium atom was located on a five-membered ring (rind D).

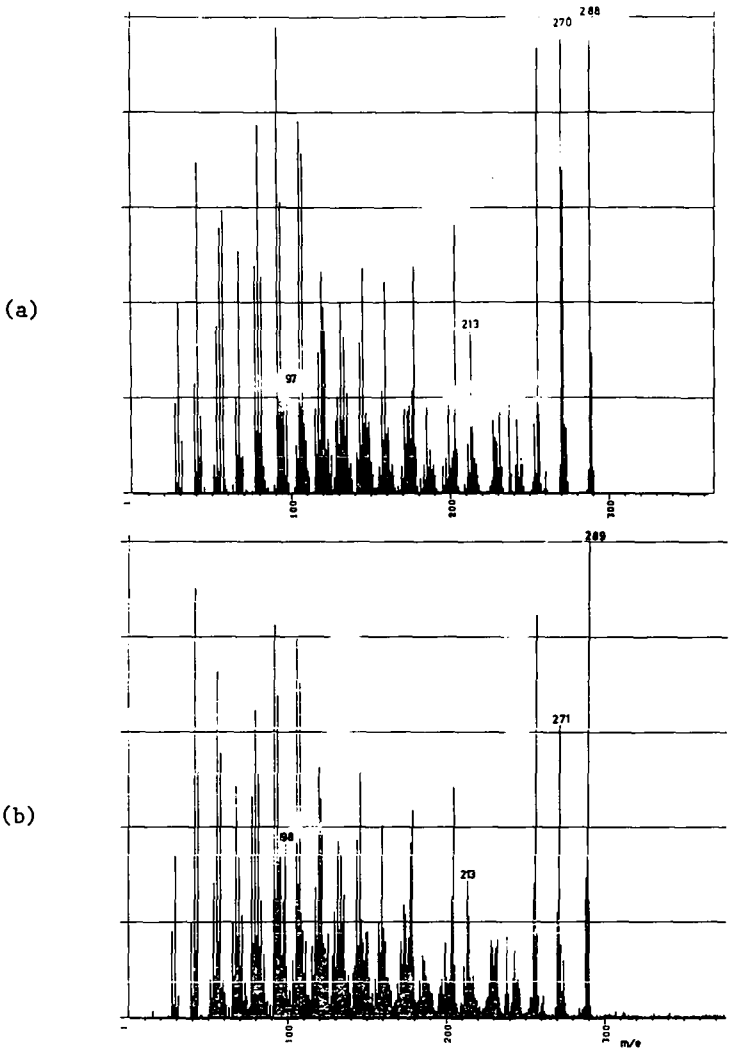

FIGURE 2

Mass spectra for unlabelled DHEA (a) and for 16α-^{2}H-DHEA (b).

Actually, a peak corresponding to a cleavage of ring D with the charge remaining on this ring occurred at m/e 98 for 16α-^{2}H-DHEA, rather than 97. In addition, the loss of a neutral fragment corresponding to 57 and 58 mass units for DHEA and 16α-^{2}H-DHEA, respectively (cleavage of the C_{13}-C_{17} and C_{14}-C_{15} bonds) led to one single intense peak at m/e 213 in both spectra, if one considers the peaks located at m/e 270 and 271 and corresponding to M-H_2O ions. From the fragmentation pattern, it was impossible to determine if the deuterium atom was located in the 15 or 16 position.

The situation was clearer for the estrone acetate sample. The molecular ion corresponded to m/e 313, one mass unit above that of the unlabelled compound. The cleavage of ring D also led to an intense ion at 213 with the charge remaining on the steroidal skeleton. This ion was common in both unlabelled and 16α-^{2}H-estrone acetates. This result confirmed the hypothesis that the deuterium atom was also in the 15 or 16 position. For this substrate, however, it was possible to confirm the 16 position for the deuterium atom.

The estrogen molecules undergo a particular fragmentation which leads to an intense ion corresponding to the following structure (Figure 4) and having a m/e value of 172 ($C_{12}H_{12}O$) (23). As both unlabelled and deuterated molecules presented this ion in their spectra, the possibility of a 15-labelled position could be discarded.

2. NMR analysis

The ^{13}C NMR spectra of the 16α-^{2}H-DHEA and 16α-^{2}H-estrone acetate were recorded (Figures 5 and 6). Both spectra, recorded under total proton decoupling, in C_6D_6 solution so that the aromatic solvent induced

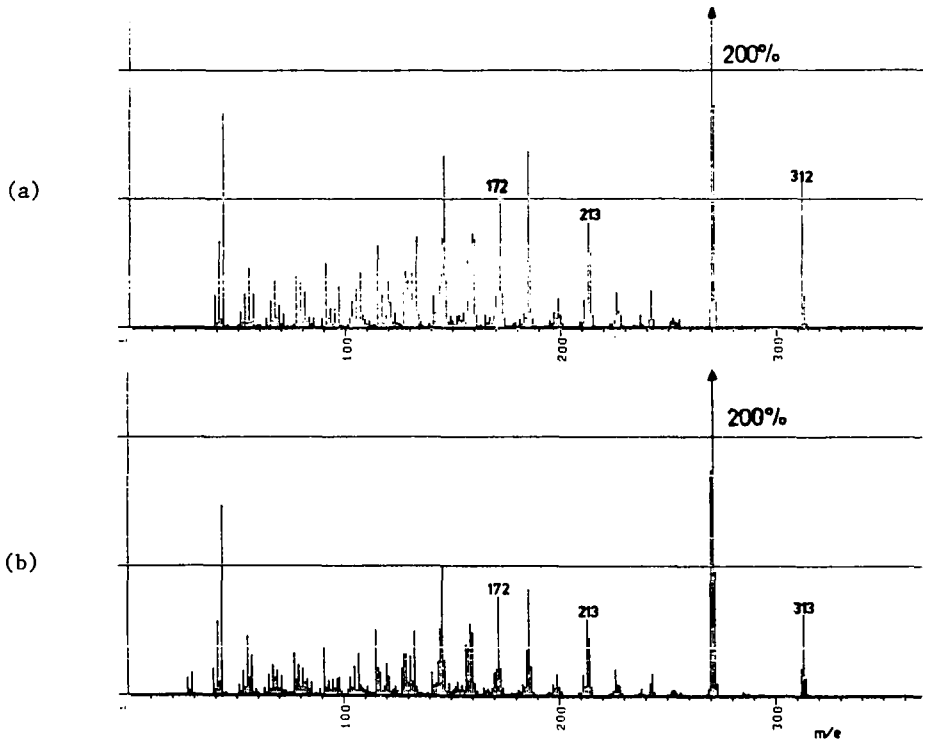

FIGURE 3

Mass spectra for unlabelled estrone acetate (a) and for 16α-^{2}H-estrone acetate (b).

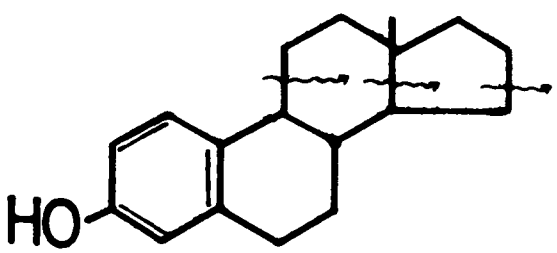

FIGURE 4

shift (20) resolved from one another the C_1, C_8 and C_{16} resonances, confirmed that deuteration took place at the 16 position. Indeed, instead of the singlet at δ = 35.4 in the case of DHEA (protium compound) or at δ = 35.4 in the case of estrone acetate ('H), a 1:1:1 triplet was found for the deuterium-bearing carbon, at δ = 35.1 (DHEA-$\underline{d}_1$ as in estrone acetate-$\underline{d}_1$). The upfield isotope shift was of the expected magnitude (21), and likewise, the observed $^1J \simeq 20$ Hz.

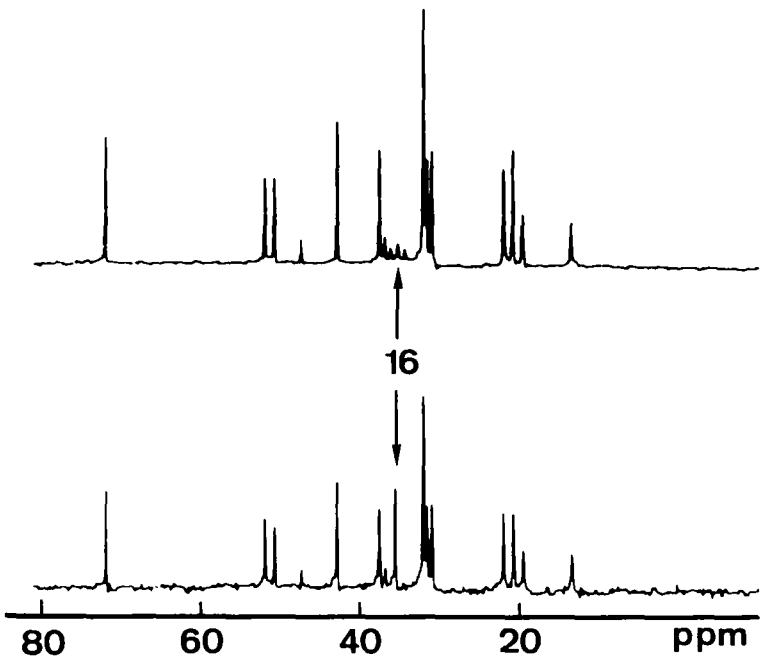

FIGURE 5: The 10 to 80 ppm portion of DHEA ^{13}C NMR spectra in a C_6D_6 solution.
Upper trace: 16α-^{2}H-DHEA. Lower trace: unlabelled DHEA. Carbon chemical shifts (δ): C_1 - 37.4; C_2, C_7, C_8, C_{12} - 31.8, 31.5, 30.8; C_3 - 71.4; C_4 - 42.6; C_9, C_{14} - 51.6, 50.3; C_{10} - 36.2; C_{11}, C_{15} - 21.5, 20.5; C_{13} - 47.1; C_{16} - 35.4 (35.1 in deuterated sample); C_{18} - 13.2; C_{19} - 19.3

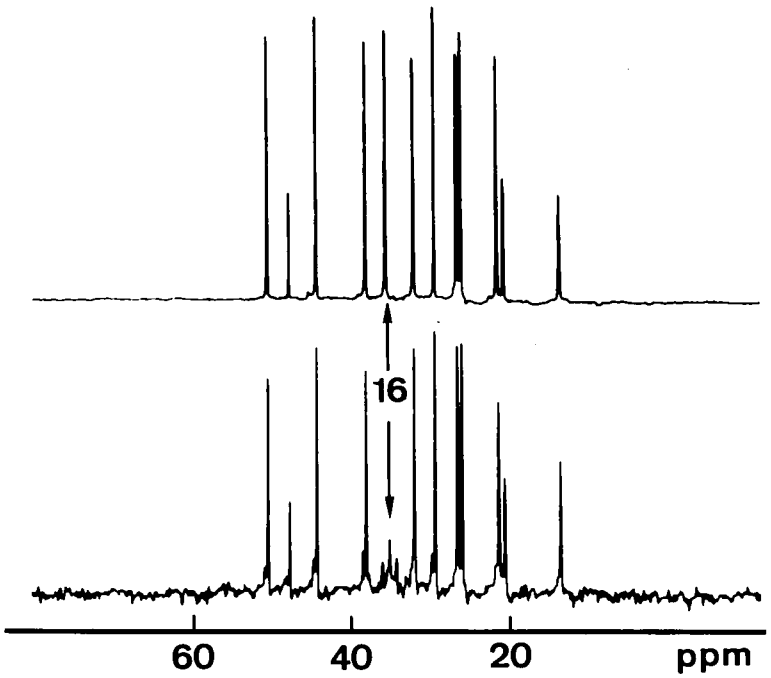

FIGURE 6: The 10 to 60 ppm portion of estrone acetate ^{13}C NMR spectra in a C_6D_6 solution. Upper trace: unlabelled estrone acetate. Lower trace: 16α-^{2}H-estrone acetate. Carbon chemical shifts (δ): C_6 - 29.4; C_7, C_{11} - 26.3, 25.8; C_8 - 37.9; C_9 - 44.1; C_{12} - 31.9; C_{13} - 47.5; C_{14}- 50.1; C_{15} - 21.3; C_{16} - 35.4 (35.1 in deuterated sample); C_{18} - 13.6

C. Microbiological methods

Since the principle of the enzymatic assay procedure for the measurement of the 16α hydroxylase activity is based upon the substitution of the tritium in the 16α position by a hydroxyl group (the tritium released being recovered in the medium as ^{3}H-water) it was also imperative to determine the exact proportion of tritium located in the 16α position. This measurement was carried out using very specific microbiological techniques. Streptomyces roseochromogenus (15) and Streptomyces halstedii (16) are two microorganisms which respectively hydroxy-

late androstenedione and estrone in the 16α position.

The specificity of the 16α labelling was determined by comparison of the $^{3}H/^{14}C$ ratios of a mixture of 16-^{3}H-androstenedione and ^{14}C androstenedione and 16-^{3}H-estrone and ^{14}C estrone with those of the corresponding 16α-hydroxy derivatives obtained after a specific enzymatic microbiological hydroxylation in the 16α position. Table 2 presents the date recorded for the various substrates. For the androgens and estrogens, 77% and 65% of the tritium were exchanged during the enzymatic reactions, respectively. The data clearly indicate that most of the tritium was located on the 16α position.

TABLE 2

Substrate	16α-Hydroxy derivatives	$^{3}H/^{14}C$ ratios	Labelling in 16α
Androstenedione		4.19	
			77%
	16α-Hydroxyandrostenedione	0.94	
Estrone		14.82	
			65%
	16α-Hydroxyestrone	5.17	

CONCLUSIONS

To quantitatively determine the main pathway for the biosynthesis of estriol and estetrol by the fetal liver microsomes, and to evaluate the best substrate for the 16α hydroxylase, it was necessary to develop a rapid, sensitive and specific assay technique, such as the tritium exchange method already applied to the measurement of different steroid hydroxylases. In order to develop such an assay, it was necessary to synthesize 16α-^{3}H compounds, androgens (DHEA, androstenedione), and estrogens (estradiol, estrone).

Since three of these substrates presented a carbonyl group adjacent

to the labelled position which is easily enolized in acid or alkaline solution, microbiological methods were developed in order to carry out oxidation and reduction reactions at neutral pH. In addition, excellent yields were obtained with a minimal release of tritium during the reactions. The understanding of the distribution of the label in the 16α position was an absolute prerequisite for the development of our enzymatic assay procedure. Chemical, physical and microbiological methods were used to guarantee the radiochemical purity of the various substrates. We measured the tritium released from these radiolabelled substrates by chemical and microbiological methods and compared the physical properties of the deuterated compounds to those of the authentic reference samples. Selective alkaline hydrolyses enabled us to determine that the tritium was located in a position adjacent to the 17 carbonyl function. Microbiological methods confirmed that most of the labelling took place in the 16α position. Mass spectrometric and ^{13}C nuclear magnetic resonance analysis of deuterated analogues clearly demonstrated that the deuterium was located in the 16 position. The specific activity obtained for all substrates was sufficient for estimating the 16α hydroxylase activities in various tissues, organs or cellular subfractions.

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ABBREVIATIONS AND TRIVIAL NAMES

I. DHEA or dehydroepiandrosterone: 3β-hydroxy-5-androsten-17-one
II. Androstenedione: 4-androstene-3,17-dione
III. Testosterone: 17β-hydroxy-4-androsten-3-one
IV. Progesterone: 4-pregnene-3,20-dione
V. Pregnenolone: 3β-hydroxy-5-pregnen-20-one
VI. Estrone: 3-hydroxy-1,3,5(10)-estratrien-17-one
VII. Estradiol: 1,3,5(10)-estratriene-3,17β-diol
VIII. Estriol: 1,3,5(10)-estratriene-3,16α,17β-triol
IX. Estetrol: 1,3,5(10)-estratriene-3,15α,16α,17β-tetrol
X. 16α-Br DHEA: 16α-bromo-3β-hydroxy-5-androsten-17-one
XI. Estrone acetate: 3-hydroxy-1,3,5(10)-estratrien-17-one 3-acetate
XII. 16α-Br estrone acetate: 16α-bromo-3-hydroxy-1,3,5(10)-estratrien-17-one 3-acetate
XIII. Estrone enol diacetate: 1,3,5(10),16-estratetraene-3,17-diol 3,17-diacetate