Ph.D. Biochemistry

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ISOLATION, ORIGIN AND METABOLISM OF NEUTRAL 15α-HYDROXYSTEROIDS IN HUMAN PREGNANCY

Labeled 15α-hydroxysteroids were prepared microbiologically. Three neutral 15α-hydroxylated steroids, namely, 15α-hydroxyprogesterone (15P), 15α-hydroxyandrostenedione and 15α-hydroxytestosterone were isolated from human late pregnancy urine. Among a large number of subjects examined, 15P could be detected only in the urine of subjects in the second and third trimester of pregnancy and it was not found in the urine of non-pregnant females and newborn infants. In two studies, ^{3}H-progesterone was introduced into the peritoneal cavity of the human fetus at the time of amniocentesis and simultaneously ^{14}C-progesterone was injected in the maternal compartment. From the glucosiduronate fraction of the urine collected following the injections, 15P was isolated which contained ^{3}H but insignificant amounts of ^{14}C indicating that 15α-hydroxylation of progesterone occurred in the fetoplacental unit and not in the mother. In a similar study in which ^{3}H-progesterone was introduced into the peritoneal cavity of the fetus and ^{14}C-15P was injected in the mother, a 0.08% conversion of progesterone to 15P was obtained. In initial studies, non-labeled 15P was administered orally to normal male subjects with or without the simultaneous intravenous injection of the labeled steroid and the urinary conjugates were hydrolyzed with Glusulase. The following six metabolites were identified: 15P, 3α, 15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 3β,15α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,15α,20β-triol and 5β-pregnane-3α,15α,20β-triol. Following the intravenous injection of labeled 15P to a pregnant and a non-pregnant female, all the metabolites described above with the exception of 5β-pregnane-3α,15α,20β-triol were shown to be normally excreted in the urine of the pregnant female but not in the urine of the non-pregnant female. The production rate of 15P in the pregnant female was calculated to be 80 μg/day.

NEUTRAL 15α-HYDROXYSTEROIDS IN MAN - G. Giannopoulos

ISOLATION, ORIGIN AND METABOLISM OF NEUTRAL 15α-HYDROXYSTEROIDS IN HUMAN PREGNANCY

by

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ABBREVIATIONS AND TRIVIAL NAMES

The following abbreviations and trivial names are used in this thesis:

dehydroisoandrosterone	3β-hydroxyandrost-5-en-17-one
dehydroisoandrosterone sulfate	17-oxo-androst-5-en-3β-yl-sulfate
16α-hydroxydehydroisoandrosterone sulfate	17-oxo-16α-hydroxyandrost-5-en-3β-yl-sulfate
androstenedione	androst-4-ene-3,17-dione
15α-hydroxyandrostenedione	15α-hydroxyandrost-4-ene-3,17-dione
11β-hydroxyandrostenedione	11β-hydroxyandrost-4-ene-3,17-dione
testosterone	17β-hydroxyandrost-4-en-3-one
15α-hydroxytestosterone	15α,17β-dihydroxyandrost-4-en-3-one
15α-acetoxytestosterone	15α-acetoxy-17β-hydroxyandrost-4-en-3-one
15α-hydroxytestosterone diacetate	15α,17β-diacetoxyandrost-4-en-3-one
adrenosterone	androst-4-ene-3,11,17-trione
progesterone	pregn-4-ene-3,20-dione
20α-dihydroprogesterone	20α-hydroxypregn-4-en-3-one
20α-acetoxyprogesterone	20α-acetoxypregn-4-en-3-one
20β-dihydroprogesterone	20β-hydroxypregn-4-en-3-one
20β-acetoxyprogesterone	20β-acetoxypregn-4-en-3-one
15α-hydroxyprogesterone	15α-hydroxypregn-4-en-3-one
15α-acetoxyprogesterone	15α-acetoxypregn-4-en-3-one
15β-hydroxyprogesterone	15β-hydroxypregn-4-en-3-one
15α-hydroxypregnenolone	3β,15α-dihydroxypregn-5-en-20-one

15-ketoprogesterone	pregn-4-ene-3,15,20-trione
16α-hydroxyprogesterone	16α-hydroxypregn-4-en-3-one
16—dehydroprogesterone	pregn-4,16-diene-3,20-dione
17α-hydroxyprogesterone	17α-hydroxypregn-4-en-3-one
isopregnanolone	3α-hydroxy-17α-pregnan-20-one
16-dehydropregnanolone	3α-hydroxy-5β-pregn-16-en-20-one
deoxycorticosterone	21-hydroxypregn-4-ene-3,20-dione
15α-hydroxydeoxycorticosterone	15α,21-dihydroxypregn-4-ene-3,20-dione
15β-hydroxydeoxycorticosterone	15β,21-dihydroxypregn-4-ene-3,20-dione
cortisone	17α,21-dihydroxypregn-4-ene-3,11,20-trione
cortisone acetate	21-acetoxy-17α-hydroxypregn-4-ene-3,11,20-trione
21-deoxycortisone	17α-hydroxypregn-4-ene-3,11,20-trione
cortisol	11β,17α,21-trihydroxypregn-4-ene-3,20-dione
6β-hydroxycortisol	6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione
9α-fluoro-hydrocortisone	9α-fluoro-11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione
aldosterone	11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al
tetrahydroaldosterone	3α,11β,21-trihydroxy-3,20-dioxo-5β-pregnan-18-al
18-hydroxytetrahydrocorticosterone	3α,11β,18,21-tetrahydroxy-5β-pregnan-20-one
Aldactone	3-(3-oxo-7α-thioacetyl-17β-hydroxyandrosten-17α-yl) propionic acid α-lactone

dexamethasone	9α-fluoro-16α-methyl-21-acetoxy-11β,17α-dihydroxypregn-1,4-diene-3,20-dione
estrone	3-hydroxyestra-1,3,5(10)-trien-17-one
estrone sulfate	17-oxoestra-1,3,5(10)-trien-3-yl-sulfate
15α-hydroxyestrone	3,15α-dihydroxyestra-1,3,5(10)-trien-17-one
15β-hydroxyestrone	3,15β-dihydroxyestra-1,3,5(10)-trien-17-one
16α-hydroxyestrone	3,16α-dihydroxyestra-1,3,5(10)-trien-17-one
estradiol	estra-1,3,5(10)-triene-3,17β-diol
15α-hydroxyestradiol	estra-1,3,5(10)-triene-3,15α,17β-triol
15β-hydroxyestradiol	estra-1,3,5(10)-triene-3,15β,17β-triol
estriol	estra-1,3,5(10)-triene-3,16α,17β-triol
15α-hydroxyestriol	estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol
DDQ	1,3-dichloro-5,6-dicyano-benzoquinone
$CDCl_3$	deuteriochloroform
TMS	tetramethylsilane
β-DPNH	Dihydro-β-Diphospho-Pyridine-Nucleotide disodium salt
Tris buffer	Tris(hydroxymethyl)-aminomethane + HCl
EDTA	ethylenediaminetetraacetic acid
N	Normality, that is, the equivalent weight of a substance in 1 litre of solution
g	acceleration due to gravity
g	gram

mg	milligram
µg	microgram
mC	millicurie
µC	microcurie
mM	millimole
µl	microlitre
ml	millilitre
dpm	disintegrations per minute
mp	melting point
mmp	mixed melting point
ppm	parts per million
^{o}C	degrees Centigrade
R_f	the ratio of the velocity of the substance under consideration to the velocity of the mobile phase in a chromatographic system.

CONTENTS

INTRODUCTION

Microorganisms were first reported to be capable of hydroxylating neutral and phenolic steroids in the 15α- (1-3) and 15β-positions (1-2). The first indication that 15α-hydroxylated steroids can be formed by mammalian tissues was the report in 1960 by Neher and Wettstein (5), who presented chromatographic evidence for the presence of 15α-hydroxytestosterone in extracts of bull testes. In 1964, Knuppen and Breuer (6) reported the *in vitro* conversion of estrone to 15α-hydroxyestrone by bovine adrenal tissue, and since that time a number of 15α- and 15β-hydroxylated estrogens has been isolated from human pregnancy urine.

During the last ten years a large number of publications has been devoted to problems of steroid metabolism in the human fetus. In this regard, studies on 15α-hydroxylated steroids are of particular interest. Although both the adult human adrenal and liver have been shown to be capable of hydroxylating estrogens in the 15α-position (7-8), such compounds have been isolated only from late human pregnancy urine (9-15). It has also been shown that the human fetus can convert estrogens to their 15α-hydroxy analogues (11,16) and available evidence suggests that 15α-hydroxylation might be a pathway of steroid metabolism which is unique to the fetus (16).

In contrast to estrogens, 15α-hydroxylation of neutral steroids by mammalian tissues has not been fully demonstrated.

Neher and Wettstein (5) presented evidence for the presence of 15α-hydroxytestosterone in extracts of bull testes. However these authors suggested that 15α-hydroxytestosterone might be of bacterial origin (private communication, Dr. A. Wettstein). When the mid-term previable human fetus was perfused with (4-^{14}C)-progesterone (17), 15α-hydroxyprogesterone could not be detected in the fetal adrenals and liver. When the present studies were initiated only 15α-hydroxylated estrogens had been found in late human pregnancy urine. Our working hypothesis was that 15α-hydroxylation of neutral steroids could occur in the human fetus later in pregnancy and an investigation was undertaken to isolate such steroids from late pregnancy urine. Before discussing the present investigations, current knowledge regarding 15α- and 15β-hydroxysteroids will be reviewed.

Isolation of 15α- and 15β-Hydroxysteroids from Urine

At the time that these investigations were initiated, 15α- and 15β-hydroxysteroids had been isolated only from human late pregnancy urine and all of them were estrogens.

The first report of the isolation of a 15α-hydroxylated steroid from human urine was published in 1965 by Knuppen et al (9), who isolated 15α-hydroxyestrone from late pregnancy urine. The concentration of this steroid in pregnancy urine was found to be approximately the same as that of 16α-hydroxyestrone, 130 μg/liter (18).

A new metabolite derived from estradiol was obtained from

neonatal and pregnancy urine by Hagen et al (10) and by Gurpide et al (11). The compound contained four acylable hydroxyl groups and formed an acetonide. One tentative structure suggested by Hagen et al (10) was 15α-hydroxyestriol. Later Fishman and Guzik (19) reported the chemical synthesis of 15α-hydroxyestriol and comparison of the synthetic tetrol with the isolated urinary metabolite showed identity in the infrared spectra, mass spectra, color reactions and R_f values in various chromatographic systems. Thus it was established that the metabolite isolated by Hagen et al and by Gurpide et al was 15α-hydroxyestriol. Recently, Zucconi et al (12) isolated 15α-hydroxyestriol from human pregnancy urine and from the urine of newborn infants and they estimated that its concentration in pregnancy urine was greater than 0.2 mg/liter.

Knuppen et al (13) isolated 2.8 mg of crystalline 15β-hydroxyestrone and 2.4 mg of crystalline 15β-hydroxyestradiol from a large pool (80 liters) of late pregnancy urine. These authors estimated that the 15β-hydroxylated estrogens were present in human pregnancy urine in similar concentrations as the corresponding 15α-hydroxylated analogues.

More recently Lisboa et al (14) isolated 5 mg of crystalline 15α-hydroxyestradiol from a pool of 100 liters of acid-hydrolyzed late pregnancy urine. The isolation of this compound from late pregnancy urine was also reported by Knuppen and Breuer (15) and its concentration in urine was estimated to range from 50 to 100 μg/liter. On the other hand, using gas liquid chromatography, it was reported that 15α-hydroxy-

estradiol is not present in detectable amounts in urine collected during the second trimester of gestation (20). This latter finding is not without precedent.

Isolation of 15α-Hydroxysteroids from Tissues

Only two reports on the isolation of 15α-hydroxysteroids from mammalian tissues can be found in the literature. In 1960 Neher and Wettstein (5) isolated 15α-hydroxytestosterone from the testicular tissue of bulls. However, the identification of this compound was based on chromatographic evidence and the authors also suggested that 15α-hydroxytestosterone might be of bacterial origin. In 1966 Zucconi et al (21) isolated from human meconium a phenolic tetrol which later was identified (19) as 15α-hydroxyestriol.

Steroid 15α-Hydroxylases

The ability of animal tissues to introduce the 15α-hydroxyl group on a preformed steroid has been studied to some extent. The experimental design employed to demonstrate these conversions has ranged from perfusion of the previable human fetus to incubations of different tissue preparations with the appropriate precursor.

In 1964 Knuppen et al (6) reported that estrone incubated with bovine adrenal homogenates was converted to 15α-hydroxyestrone. The same authors later reported the conversion of estrone and estradiol to 15α-hydroxyestradiol by human adrenal tissue slices (7) and by human liver slices (8).

In 1965 Schneider (22) demonstrated the conversion of deoxycorticosterone to 15α- and 15β-hydroxydeoxycorticosterone by liver slices of the American bullfrog, and Levy et al (23) isolated 15α-hydroxyestradiol from bovine adrenal glands perfused with ^{14}C-estradiol. The isolation of 15α-hydroxyestradiol from the liver of previable human fetuses perfused with labeled estrone and estrone sulfate was reported by Schwers et al (16). This metabolite was also isolated from the fetal liver and the placenta following the injection of labeled estrone and estradiol into the intact feto-placental circulation at the time of laparotomy (24). Mancuso et al (25) isolated 15α-hydroxyestradiol from the liver of previable human fetuses perfused with labeled androstenedione and testosterone but 15α-hydroxyandrostenedione or 15α-hydroxytestosterone was not isolated. It is now well established that the human fetal liver possesses a very active steroid 15α-hydroxylase.

Formation and Metabolism of 15α- and 15β-Hydroxysteroids

Present evidence suggests that there are at least two pathways by which 15α-hydroxylated estrogens are formed; direct 15α-hydroxylation of preformed phenolic precursors, or 15α-hydroxylation and aromatization of C-19 neutral steroids. Whether aromatization or 15α-hydroxylation of neutral steroid precursors is the first step in the formation of 15α-hydroxylated estrogens is not clear. It was mentioned earlier that when previable human fetuses were perfused with labeled androstene-

dione and testosterone only 15α-hydroxyestradiol was isolated from the fetal liver and 15α-hydroxyandrostenedione or 15α-hydroxytestosterone could not be detected (25). On the other hand, in our laboratories Stern, Givner and Solomon (26) showed a 30-40% conversion of labeled 15α-hydroxyandrostenedione to 15α-hydroxyestradiol following incubation of the labeled precursor with the 10,000 x g supernatant fraction of human placental tissue and YoungLai and Solomon (27) have isolated labeled urinary 15α-hydroxyestriol following the intravenous injection of labeled dehydroisoandrosterone sulfate and 16α-hydroxydehydroisoandrosterone sulfate to a pregnant female.

Following the intramuscular administration of estradiol to infants born with multiple malformations, Hagen et al (10) isolated from the urine a metabolite which was later identified as 15α-hydroxyestriol (19). This compound was also detected by Gurpide et al (11) in the urine of pregnant women with erythroblastosis fetalis following the simultaneous administration of tritium labeled estradiol into the peritoneal cavity of the fetus at the time of amniocentesis and the injection of ^{14}C-labeled estradiol in an antecubital vein of the mother. The isolated 15α-hydroxyestriol contained tritium but little or no ^{14}C, thus demonstrating its feto-placental origin. In the same studies labeled estradiol was administered to non-pregnant women and labeled 15α-hydroxyestriol was not detected in the urine. Labeled estriol injected intra-amniotically served as a precursor of 15α-hydroxyestriol. On the

other hand, labeled 15α-hydroxyestriol was not found in the urine after the administration of labeled estriol to a non-pregnant woman. In some pregnant subjects maternally administered estradiol or dehydroisoandrosterone sulfate was converted to a small extent to 15α-hydroxyestriol. When a mixture of tritiated estriol and ^{14}C-estradiol was administered intra-amniotically to a pregnant subject (28), 15α-hydroxyestriol containing both ^{3}H and ^{14}C was isolated from the urine. The minimal percent conversions of estradiol and estriol to 15α-hydroxyestriol was 12 and 0.7, respectively. The results obtained in these studies also suggested that the conversion of estradiol to 15α-hydroxyestriol occurs only partially via estriol.

Jirku et al (29) injected labeled estrone sulfate to a non-pregnant female and they isolated labeled 15α-hydroxyestrone from the sulfate fraction of the bile. The 15α-hydroxyestrone "sulfate" comprised about 10% of the radioactivity in the biliary sulfate fraction which comprised about 15% of the total radioactivity excreted in the bile. One third of the total radioactivity excreted was detected in the bile. However, 15α-hydroxylated estrogens have not as yet been isolated from the urine of non-pregnant females.

There is a single report in the literature on the metabolism of 15α-hydroxylated estrogens. In 1966, Knuppen et al (13) studied the metabolism of 15α-hydroxy- and 15-oxo-estrogens <u>in vitro</u> using human liver slices. A number of products was

isolated, two of which were identified as 15β-hydroxyestrone and 15β-hydroxyestradiol. These authors suggested that 15β-hydroxylated estrogens arise either by direct 15β-hydroxylation of estrone and/or estradiol or by partial oxidation of 15α-hydroxyestrogens to form the corresponding 15-oxo- compounds and the metabolic reduction of the latter to 15β-hydroxyestrogens.

MATERIALS

Purification of Solvents

Acetic anhydride (Fisher certified) was distilled over fused sodium acetate in an all glass system and stored under anhydrous conditions in a desiccator.

Pyridine (Fisher certified) was distilled over lumps of barium oxide under anhydrous conditions. The pure reagent was stored in a desiccator.

Absolute ethanol (Gooderham and Worts) conforming to the specifications of the British and U.S. Pharmacopoeia, was used directly as purchased.

Tetrahydrofuran (Fisher certified) was refluxed for a minimum of two hours over KOH pellets and then distilled. This solvent was purified immediately before use.

Benzene (Analytical grade, Mallinckrodt) was distilled over KOH pellets prior to use. Dry benzene was prepared by distilling benzene with phosphorous pentoxide under anhydrous conditions. The dry reagent was stored in a desiccator.

Diethyl ether (Analytical grade, Mallinckrodt) was distilled over KOH pellets prior to use and was stored in the cold for short periods of time in brown bottles.

Chloroform (Analytical grade, Mallinckrodt) was distilled over potassium carbonate and preserved by the addition of ethanol (1% solution).

The solvents listed below were all distilled prior to

use without any additional treatment:

Tertiary Butanol (Practical grade, Distillation Products Ltd.)

Acetone (Analytical grade, Mallinckrodt)

Cyclohexane (Practical grade, Distillation Products Ltd.)

Iso-Octane (Practical grade, Distillation Products Ltd.)

Ethyl acetate (Fisher certified)

Methanol (Fisher certified)

Methylene chloride (Fisher certified)

Skellysolve B (Skelly Oil Co.)

Toluene (Fisher certified).

All other reagents and chemicals mentioned in the investigations to be described were used as purchased.

Steroids

The following steroids were used as standards and their source is indicated:

16α-Hydroxyprogesterone (Squibb Institute for Medical Research, New Brunswick, New Jersey)

15α-Hydroxyprogesterone (a gift of Dr. P.A. Diassi, Squibb Institute)

15α-Hydroxyandrostenedione (a gift of Dr. P.A. Diassi)

15α-Hydroxytestosterone (a gift of Dr. P.A. Diassi)

Progesterone (a gift of Dr. A. Zaffaroni, Syntex)

Androstenedione (a gift of Dr. A. Zaffaroni).

The melting point and infrared spectrum were obtained on

each standard prior to use. In some instances the standards were purified by chromatography on small columns and recrystallized prior to use.

Radioactive steroids were purchased from New England Nuclear Corp., Bostnn, Massachusetts and were checked for purity as described in a separate section of this thesis.

Enzymes and Cofactors

Dihydro-β-Diphospho-Pyridine Nucleotide Disodium salt (β-DPNH) was purchased from Sigma Chemical Co.

20β-Hydroxy Steroid Dehydrogenase, Type 11 (from Streptomyces hydrogenans) was used as a suspension in 2.2M ammonium sulfate, 5 mg/ml (Sigma, Lot 97B-0490). The enzyme was always stored at 0-5°C. One mg of the enzyme reduces 12 μmoles of cortisone per minute with β-DPNH at pH 7.6 and 25°C.

Ethylenediaminetetraacetic Acid was used as purchased (EDTA, Fisher Scientific Co.).

Special Reagents

Phosphomolybdic acid (Fisher certified): A 10% (w/v) solution in absolute ethanol was prepared. This solution was stable for long periods of time when refrigerated in a tightly stoppered dark container.

Alumina (200 mesh; Harshaw Chemical Co., Cleveland, Ohio) was washed and deactivated according to the procedure described by Solomon et al (30). The alumina was refluxed with ethyl

acetate for five days. It was then washed with methanol, with water for two days, and again with methanol. After drying in the oven at 120°C it was deactivated by the addition of 5 ml of water per 100 g alumina and stored in tightly stoppered bottles.

Celite (Johns-Manville, No. 545), was first washed with 50% hydrochloric acid for 24 hours, then with tap water for 72-100 hours, and with distilled water, and finally with methanol. It was then air-dried at room temperature for 48 hours, or in an oven at 80-90°C for 18 hours and stored in brown bottles.

Silica gel (100-200 mesh; Davison Chemical Co., Baltimore, Maryland) was purchased in 5 lb. lots, and used directly.

METHODS

Measurement of Radioactivity

Aliquots of samples to be counted were evaporated under nitrogen in 5 dram vials (Wheaton Glass Co., Millville, N.J.) and dissolved in 10 ml toluene containing 0.3% (w/v) of 2,5-diphenyloxazole (PPO) and 0.01% (w/v) of 1,4-bis [2-(5-phenyloxazolyl)]-benzene (POPOP). Samples which were insoluble in toluene were dissolved in 2 ml of methanol prior to the addition of 10 ml of toluene phosphor. Radioactivity in aqueous solutions, such as urine, was measured by dissolving a 1 ml aliquot in 15 ml of a dioxane solution which contained 0.7% (w/v) of PPO, 0.06% (w/v) of POPOP and 10% (w/v) of naphthalene. All samples were counted for a time sufficient to give a standard deviation of no more than 5% in the case of quenched samples, and 2% in the case of unquenched samples. A three channel Packard Tri-carb liquid scintillation spectrometer, Model 3002, was used for counting.

When a single isotope was counted, the settings of the instrument were as follows: The amplifier gain of the green and red channels was set at 60% and the pulse height discriminators at 50 to infinity. At these settings ^{3}H was counted with an efficiency of 35% and ^{14}C with an efficiency of 85%. In the presence of methanol the efficiency of counting was 18% and 80% for ^{3}H and ^{14}C respectively.

For the simultaneous counting of ^{3}H and ^{14}C, the red

channel gain was set at 30% with pulse height discriminators at 50 to 450; the gain on the green channel was set at 40% with the discriminators at 700 to infinity. Efficiency of counting at these settings was 28% for ^{3}H and 64% for ^{14}C. When methanol was in the counting vials, the red channel gain was set at 40% with the discriminators at 50 to 300, and the green channel had a gain setting of 50% with the discriminators at 550 to infinity. Efficiency of counting at these settings was 15% for ^{3}H and 53% for ^{14}C.

The absolute efficiency of counting was calculated with the use of ^{3}H and ^{14}C labeled hexadecane standards obtained from The Radiochemical Centre, Amersham, England. Counting vials were washed with detergent, then rinsed thoroughly with water and with methanol. After drying in an oven and equilibrating at room temperature the vials were weighed to a constant weight. Aliquots of ^{3}H and ^{14}C hexadecane (dissolved in absolute ethanol) were then transferred to the vials and dried under a stream of nitrogen. The vials were then kept in a desiccator for a few hours before weighing. This process of desiccating and weighing was continued until a constant weight was obtained. The total dpm of each sample was computed from the weight of the hexadecane and its specific activity which was 4.0×10^{3} dpm/mg for ^{3}H and 2.13×10^{3} dpm/mg for ^{14}C. The ^{3}H and ^{14}C standards were counted with each set of samples.

In simultaneous counting of two isotopes, the total ^{3}H

and ^{14}C counts were calculated using the discriminator ratio method of Okita et al (31) as modified by Ulick (32). The following equations were used:

$$^{3}H = N_1 - \frac{N_2}{b}$$

and $$^{14}C = N_2 - N_1 a$$

where N_1 = total counts in the red channel

N_2 = total counts in the green channel

$$a = \frac{^{3}H \text{ in the green channel}}{^{3}H \text{ in the red channel}}$$

$$b = \frac{^{14}C \text{ in the green channel}}{^{14}C \text{ in the red channel}}.$$

Every set of vials was counted with ^{3}H and ^{14}C standards in order to determine the "a" and "b" ratios. The optimum conditions for double label counting in the absence of methanol were obtained with an "a" ratio of approximately 0.008 and a "b" ratio of 3.05; in the presence of methanol, the "a" and "b" ratios were approximately 0.017 and 4.08 respectively. All counts were converted directly to dpm.

Counts of samples which only contained a single label were corrected for quenching with the use of ^{3}H and ^{14}C labeled toluene as internal standards. Vials were recounted after addition of a known amount of radioactivity in 0.1 ml or 0.2 ml of toluene. Comparison of the increment of counts with the actual number of counts added, provided a ratio which was used to correct the original quenched sample count. Samples

containing both ^{3}H and ^{14}C were recounted after the addition of ^{3}H internal standard and again after the addition of ^{14}C internal standard. The values of "a" and "b" were calculated from the increments in the two channels following addition of the two standards.

Melting Point Determinations

All melting points were determined with a Kofler block (H.O. Post Scientific Co. Inc., New York) and were corrected by reference to a standard curve based on the observed melting points of a series of primary standards (Fisher Scientific Co.)

Spectral Analysis

Infrared spectra were obtained with the use of a Perkin-Elmer Model 221 Spectrophotometer. Samples were prepared as described by Roberts et al (33) and were examined as 1% solutions in CS_2 whenever possible. Samples insoluble in CS_2 were examined as dispersions in KBr discs, using 200-500 µg of steroid and 20 mg of KBr (Powdered, I.R. 6890, Infrared Quality, Harshaw Chemical Co., Cleveland 6, Ohio). In some instances only microamounts (20-60 µg) of a compound were available for infrared analysis and these were examined in KBr (10 µg/2 mg KBr) with the aid of a Perkin-Elmer 6x microsampling unit (Model 186-0011).

Mass spectra analyses were performed through the courtesy

of Drs. L. Durham and C. Djerassi, Department of Chemistry, Stanford University, California, and in one instance by Dr. G. Slomp, the Upjohn Company, Kalamazoo, Michigan.

Nuclear magnetic resonance spectroscopy was performed through the courtesy of Drs. L. Durham and C. Djerassi, and in one instance by Dr. G. Slomp. The nuclear magnetic resonance spectra (NMR) were run on a Varian HR-100 spectrometer. Deuteriochloroform ($CDCl_3$) was used as the solvent with tetramethylsilane (TMS) as an internal standard. Samples were examined as $CDCl_3$ solutions in a glass microcell (NMR Specialties) of approximately 50 µl volume. Chemical shift measurements were obtained from individual side-band measurements, using TMS as reference standard. In some instances a Varian HA-100 spectrometer was used, and since the instrument was field-frequency locked, the spectra were displayed on precalibrated charts.

Chromatography

Alumina Column Chromatography: Alumina (200 mesh) was prepared for use as previously described. The weight of adsorbent used was approximately 100 times that of the weight of extract to be purified. The columns were prepared by slowly pouring the alumina into a small glass column containing the developing solvent, usually Skellysolve B. At the same time, the column was tapped vigorously to eliminate air bubbles. Fractions of 1 ml per g of alumina were collected. Steroids

were eluted with increasing concentrations of benzene in Skellysolve B or with increasing concentrations of absolute ethanol in benzene. The column fractions were combined on the basis of a visual examination of the residue in each flask or on the basis of the radioactivity in each fraction.

Celite Column Partition Chromatography: Celite, prepared as described above, was used in the ratio of 0.5 to 1.0 g per mg of extract. All columns used had a ratio of height to diameter of 20 or more. The Celite was stirred in a Waring Blender with sufficient mobile phase to exclude air; stationary phase, 0.5 ml per g of Celite, was added slowly to the mixture, then the Celite was added to a glass chromatographic column and packed tightly with a Martin Packer (34). When possible, samples were dissolved in a minimum volume of mobile phase and the solution was applied to the top of the column. Those samples not readily soluble in mobile phase were dissolved in stationary phase and mixed with Celite in the same proportions used to pack the column, and the charge was applied to the top of the column. The effluent was collected in tubes using an automatic fraction collector (Buchler Instruments, N.J.). Holdback volumes (HBV) of columns were determined by measuring the volume of solvent required to elute the dye, Sudan IV, as described by Johnson (35) and varied from 1.2 to 1.8 ml per g of Celite. All Celite columns were developed at room temperature. The effluent from the columns was collected at the rate of 0.2 to 0.4 ml/g of Celite per hour, and analysed by measuring

the radioactivity of suitable aliquots from each fraction.

Paper Chromatography: Sheets of Whatman No. 1 or No. 3MM filter paper 17x57 cm were used throughout. Chromatography on paper was accomplished by either one of two methods. When Bush type (36) systems were used, the sample was applied on the starting line of the paper which was then equilibrated in the chromatography tank for a minimum of four hours before the addition of mobile phase. When Zaffaroni type (37) systems were used, the paper was impregnated with stationary phase by dipping in 50% solution of stationary phase in methanol and blotted twice just prior to the application of the samples in order to remove excess stationary phase. No equilibration was necessary when such systems were employed.

Ultraviolet absorbing steroids were located on paper with the aid of a viewing box (Chromato-Vue, Ultraviolet Products Inc., San Gabriel, Calif.). Other types of steroids were visualized by spraying a 1 cm strip of the chromatogram with phosphomolybdic acid followed by heating of the papers to about 90°C for 1-2 minutes, or until a blue spot appeared against a yellow background. This method of detecting steroids on paper was originally devised by Kritchevsky and Kirk (38). Radioactive steroids were located with the aid of a Packard Model 7200 strip-scanner.

Areas of paper containing the steroid to be eluted were cut in small squares, covered with methanol and allowed to stand for 2-15 hours. After filtering the solvent, the paper

squares were washed twice with additional amounts of methanol, and the combined methanol extract was evaporated in vacuo. When propylene glycol was present, the residue after evaporation of methanol was dissolved in ethyl acetate and it was washed three times with 1/5 volume water to remove any traces of this stationary phase. The ethyl acetate was dried over Na_2SO_4 and evaporated under vacuum at 40°C.

Silica Gel Column Chromatography: Urinary extracts were first chromatographed on large silica gel columns. A slurry of silica gel in methylene chloride was transferred to a glass column which was tapped vigorously to ensure even settling of the support and the exclusion of air bubbles. The sample to be chromatographed was dissolved in methylene chloride and applied to the top of the column. The column was developed with methylene chloride or with ethanol in methylene chloride depending on the nature of the extract. The effluent from the column was collected at the rate of 30-40 ml/hr when large columns were used. Individual fractions sometimes varied from 8-12 ml but most fractions were about 10 ml. A timer was employed to regulate the volume of the fractions collected. Aliquots of fractions were removed for the determination of radioactivity and individual fractions from each column were combined according to the plot of radioactivity versus fraction number. When large columns (100-300 g) were employed an aliquot from every fourth fraction was taken for counting.

Small silica gel columns were also used for further purification of the steroids eluted after paper chromatography. These columns were prepared in the manner described above except that 1 ml fractions per g of adsorbent were collected.

Thin Layer Chromatography: Silica gel G (Merck) was mixed with a volume of water equivalent to twice the weight of the silica gel, and spread on 20x20 cm glass plates to a depth of 1 mm, using a Research Specialties Co. spreader. The plates were dried in air for one hour or more, and then heated to 120°C for one-half hour. Samples were spotted on a line 2 cm from one edge. Chromatograms were developed by ascending solvent flow in small glass tanks with glass covers without equilibration.

Steroids were located on the thin layers by their absorption of UV light or by spraying with phosphomolybdic acid as previously described for paper chromatograms. Immediately after chromatography, the silica gel to be eluted was scraped off the plate with a blade and transferred to a medium porosity sintered glass funnel of 100 ml capacity. The support was covered with ethyl acetate, stirred, and allowed to stand for 5 to 10 minutes. The ethyl acetate was then filtered off with the aid of suction and the extraction was repeated twice, after which the combined ethyl acetate was evaporated at 40°C.

Solvent systems used in all of the chromatographic se-

parations to be described in the thesis are shown in Table 1.

TABLE 1

Solvent Systems Used in Chromatography

System	Type	Solvents
A	PPC*	Toluene-propylene glycol
B	PPC	Skellysolve B:methanol:water (10:9:1)
C	PPC	Iso-octane:toluene:methanol:water (5:5:7:3)
D	PPC	Iso-octane:methanol:water (10:9:1)
E	CPC**	n-Heptane:ethyl acetate:methanol:water (10:10:13:7)
F	CPC	n-Hexane:ethyl acetate:methanol:water (10:10:13:7)
G	PPC	Benzene:cyclohexane (1:1) - propylene glycol
H	PPC	Benzene:cyclohexane (1:1) - ethylene glycol
J	PPC	Benzene:methanol:water (20:11:9)
K	TLC***	Benzene:ethanol (1:1)
L	PPC	Toluene:ethyl acetate: methanol:water (9:1:6:4)
M	PPC	Chloroform:ethyl acetate (5:1) - formamide
N	TLC	Chloroform:ethanol (9:1)
O	TLC	n-Hexane:ethyl acetate (1:1)
P	PPC	Iso-octane:t-butanol:methanol:water (10:2:7:1)
Q	TLC	Benzene:ethyl acetate (2:1)

*PPC - Paper Partition Chromatography
**CPC - Celite Column Partition Chromatography
***TLC - Thin Layer Chromatography

Preparation of Steroids for Injection

Whenever possible, labeled steroids were stored in benzene:methanol (4:1) solutions in the refrigerator in order to minimize the dangers of self decomposition. Prior to injection suitable aliquots were transferred to sterile vials, using sterile pipettes. Solvents were evaporated under nitrogen and the residues were dissolved in 0.5 ml of absolute ethanol. Immediately before injection, 10 ml of sterile isotonic saline was added to the vials and the mixture was then injected slowly into the antecubital vein of the subject from a 20 ml syringe. The syringe was washed once by drawing the subject's blood which was re-injected. After the injection the syringe, needle and vial were washed with saline and with ethyl acetate, and the radioactivity measured in these washings. This radioactivity was subtracted from the radioactivity originally prepared for the injection.

In some instances a more direct approach was employed to determine the formation and metabolic fate of a steroid in late pregnancy. This approach involved the administration of the tritium labeled steroid into the peritoneal cavity of the fetus during a transfusion *in utero*, for erythroblastosis fetalis, and the simultaneous injection of the ^{14}C-labeled steroid into an antecubital vein. Maternal urine was then collected and the metabolites isolated. The injection of the steroids and collection of urine was done by Dr. J.M. Bowman, R_h Laboratory, Winnipeg, Canada. The urine was then frozen

and sent to Montreal. The steroid to be administered into the peritoneal cavity of the fetus was transferred from the vial with a syringe and mixed with the packed donor cells. The vial was rinsed with 4 ml saline. This was drawn into the syringe and also added to the blood. Usually the steroid was mixed with 150 ml of blood which was transfused slowly. At the end of the transfusion, the remainder of the blood (some 30-40 ml) was sent together with the vial, the syringe, and the plastic tubing. The syringe, needle and vial were washed with saline and with ethyl acetate, and the residual radioactivity was measured. The blood was transferred into a large beaker and mixed with a known amount of steroid labeled with ^{14}C. Ethyl acetate was then added, the mixture stirred, and the ethyl acetate was removed after equilibration. Washing of the blood with ethyl acetate was repeated 3-4 times and the radioactivity present in the combined ethyl acetate washings was measured and corrected for losses using the percent recovery of the labeled steroid added in the blood. The radioactivity adsorbed on the plastic tubing was estimated by counting one cm portions of the tubing in toluene phosphor. The total radioactivity recovered from the vial, syringe, needle, blood and the tubing was then subtracted from the radioactivity originally prepared for the injection.

Hydrolysis of Urinary Conjugates

Urines not processed immediately after collection were

stored in the frozen state.

In order to hydrolyze the steroid sulfates, a modification of the solvolytic procedure of Jacobsohn and Lieberman (44) was employed. Each day's urine was adjusted to pH 1 with sulfuric acid. After addition of sodium chloride, 20% (w/v), the urine was divided in five equal aliquots and each one was extracted with a volume of freshly distilled tetrahydrofuran equal to the total volume of urine. The tetrahydrofuran was filtered through glass wool and to it was added 0.11 ml of 60% perchloric acid per 100 ml of solvent and the solution was left overnight in the dark at 37°C. The tetrahydrofuran solution was then neutralized with 0.5 ml of concentrated ammonium hydroxide per 100 ml solution and the solvent was evaporated in vacuo, to yield an aqueous phase which was extracted with ethyl acetate. A volume of ethyl acetate 1.5 to 2 times that of the aqueous phase was divided among 3 separatory funnels in the ratio of 2:1:1. The aqueous phase was then passed through the three funnels in sequence, using about 1/5 of the solution at a time. The ethyl acetate was then combined, washed with 0.5N NaOH to remove pigments andacids, and with water until the water washes were neutral. The ethyl acetate was then dried over Na_2SO_4, filtered and evaporated in vacuo at 40°C. The residue thus obtained constituted the steroids excreted as "sulfates" and it will be referred to as the sulfate fraction.

The residual aqueous phase plus the alkali and water washes

from the above extraction contained the steroid glucosiduronates. In order to hydrolyze the conjugates, the combined aqueous phase was adjusted to pH 4.7 with glacial acetic acid, mixed with 5 ml of 2M acetate buffer pH 4.7 per 100 ml, and to this solution was added 40 mg of powdered β-glucuronidase preparation (Baylove Chemicals, Musselburgh, Scotland, two million Fishman units per gram) per 100 ml of solution. Methylene chloride (10 ml) was added to the solution as preservative. After incubation for 5 days at 37°C a neutral extract was prepared as previously described for the sulfate fraction. This extract contained the steroids excreted as glucosiduronates and also those steroids excreted as double conjugates with sulfuric and glucuronic acids (e.g. 3-sulfate-15-glucosiduronate) and it will be referred to as the glucosiduronate fraction.

In some cases, simultaneous enzymatic hydrolysis of sulfates and glucosiduronates was accomplished with the use of Glusulase (Endo Laboratories, New York), a mixture containing 100,000 units of β-glucuronidase and 50,000 units of sulfatase per ml. Urine was adjusted to pH 5.2 with glacial acetic acid, and 5 ml of 1.2N sodium acetate buffer plus 0.5 ml of the enzyme mixture were added per 100 ml of urine. Methylene chloride (10 ml) was added as a preservative and the mixture was incubated at 37°C for 3-5 days. A neutral ethyl acetate extract was then prepared as previously described.

Preparation of Derivatives

Acetates: Steroids to be acetylated were dissolved in two parts of pyridine and one part of acetic anhydride in glass stoppered tubes, and left in the dark at room temperature for about 18 hours. Steroids acetylated with labeled acetic anhydride were dissolved in equal volumes of pyridine and a 20% solution of acetic anhydride in benzene and the mixture was incubated overnight at 37°C. At least a 0.2 molar excess of acetic anhydride per mole of steroid was used. Acetylations were stopped in two ways. In one procedure, the tube containing the reactants was placed in a water bath at 40°C and the solvents were evaporated under a stream of nitrogen. Several portions of benzene:methanol (1:1) were added and each was evaporated until the odor of pyridine could not be detected. In the second method, the reaction mixture was transferred to ice-water containing 5% 6N sulfuric acid, and the steroid acetate was extracted with ethyl acetate. The organic phase was washed five times with 6N sulfuric acid, three times with 1N sodium hydroxide and with distilled water until neutral, dried over Na_2SO_4, filtered and evaporated *in vacuo*.

Sodium borohydride reduction: Reduction with $NaBH_4$ was performed by the method described by Norymberski and Wood (39) with minor modifications. A 0.4% solution of the steroid was prepared in methanol. This solution was cooled in ice-water to 4°C. To it was added a 1.6 molar excess of $NaBH_4$ and the

reaction allowed to proceed for one hour. The reaction was stopped by the addition of 6 drops of glacial acetic acid and the methanol was evaporated to give a residue which was dissolved in ethyl acetate and water. Then the organic phase was washed with 0.1N sodium hydroxide, water, dried over Na_2SO_4 and evaporated in vacuo.

Preparation of 15α-Acetoxy-20β-hydroxypregn-4-en-3-one: An aliquot of 15α-acetoxyprogesterone (3.4 mg) was dissolved in 0.4 ml ethanol and the solution was added slowly to 20 ml of 0.1M Tris buffer pH 7.6 with EDTA (2.7×10^{3}M) in a 125 ml Erlenmeyer flask. Following the addition of 194 mg β-DPNH and 0.2 ml (1.0 mg) of 20β-hydroxysteroid dehydrogenase, the mixture was incubated at 25°C and 180 cycles per minute in a Gyrotory Shaker (New Brunswick Scientific Co., New Jersey). After two hours of shaking, the reaction was stopped with 50 ml of distilled water and the steroids were extracted with ethyl acetate to give 2.8 mg of residue. It was chromatographed on a thin layer plate using system Q and two ultraviolet-absorbing bands were observed at average distances of 4.8 and 6.3 cm. The less polar band corresponded in mobility to 15α-acetoxyprogesterone standard. The more polar band was eluted to give a residue (2.3 mg) which was crystallized from acetone-Skellysolve B to yield 1.5 mg of coarse needles, mp 192-193°C. Its infrared spectrum (CS_2) indicated the retention of the acetate group (1738 and 1240 cm^{-1}), the presence of the Δ^4-3-ketone function, the loss of the 20-ketone group (1710 cm^{-1}) and the

appearance of a hydroxyl band (3590 cm^{-1}). This infrared spectrum was identical to the spectrum of the material obtained following $NaBH_4$ reduction of 15α-acetoxyprogesterone and oxidation of the resulting product with the DDQ reagent as described below. The material obtained after incubation of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase was therefore identified as 15α-acetoxy-20β-hydroxypregn-4-en-3-one.

Dichlorodicyanobenzoquinone Oxidation: A derivative of 15α-acetoxyandrostenedione and 15α-acetoxyprogesterone was prepared by reduction with sodium borohydride and subsequent oxidation of the allylic alcohol at C-3 with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), according to a modification described by Ruse and Solomon (40) of the original method by Burn et al (41). In a typical example a total of 44 mg of 15α-acetoxyprogesterone was reduced with $NaBH_4$ as described above. The products of reduction were dissolved in 3 ml of freshly distilled dioxane contained in a glass stoppered tube to which was added 33 mg of DDQ reagent. The reaction mixture was left in the dark at room temperature for 27 hours and the hydroquinone formed was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate and the organic phase was washed first with 0.5N NaOH until all pigments had been removed, and then with water, dried over Na_2SO_4, filtered and evaporated *in vacuo*. A residue weighing 360 mg was chromatographed on a 5 g alumina column, and elution with

benzene yielded 30.9 mg of crystalline material. Crystallization from ether-methanol gave 25.6 mg of coarse needles, m.p. 192-193°C, which were UV positive when a solution of the crystals was applied on paper. Its infrared spectrum (CS_2) was identical to that of 15α-acetoxy-20β-hydroxypregn-4-en-3-one prepared with 20β-hydroxysteroid dehydrogenase as described above. A mixture of the $NaBH_4$ reduction product and the material prepared with 20β-hydroxysteroid dehydrogenase did not show depression of the m.p. (mmp: 193-194°C). Similarly, the structure of the derivative prepared by $NaBH_4$ reduction and DDQ oxidation of 15α-acetoxyandrostenedione will be 15α-acetoxytestosterone. Direct evidence for the formation of the latter will be presented in the experimental section.

Recovery of Steroids from Counting Vials

In some instances material taken for counting had to be recovered in order to continue with an identification or purification process (42). The contents of the counting vials were dried under nitrogen and chromatographed on thin layer plates in system ethyl acetate:n-hexane (1:1). The PPO and POPOP usually migrated with the solvent front while the steroid was slower in mobility. The steroid was then eluted with ethyl acetate.

Standardization of Labeled Acetic Anhydride

Three Batches of (1-^{14}C)-acetic anhydride (Batches #1,

#2 and #3), specific activity 1 mC/mM, were received from New England Nuclear Corporation. These solutions contained 1 mC (10.2 mg) of the reagent in 0.04 ml of benzene and they were used directly. Two dilutions of Batch #1 (25-fold and 125-fold) with 20% acetic anhydride in benzene gave solutions 1 and 2. Two Batches of ^{3}H-acetic anhydride (Batches #4 and #5), specific activity 10 mC/mM and 100 mC/mM, respectively, containing 1 mC (10.2 mg) of the reagent in 0.04 ml of benzene were also used. The specific activity of the acetic anhydride in each solution was determined by the acetylation of 15-25 mg deoxycorticosterone (DOC) and the measurement of the specific activity of the resulting acetate (DOCA). After acetylation of known amounts of non-labeled steroid the products were chromatographed on alumina columns prior to crystallization. Constant specific activity of each acetate was achieved as shown in Table 2.

Determination of Endogenous Specific Activities (dpm/mg) of Isolated Metabolites

Following initial chromatography of urine extracts on a silica gel column, the material within each peak was further purified and partially identified by chromatography on a Celite column and/or paper. To facilitate crystallization of the isolated steroids after final chromatography, they were percolated through small silica gel or alumina columns as previously described. For the determination of the endogenous specific

TABLE 2

Standardization of Labeled Acetic Anhydride*

		Specific Activity (dpm/μg DOCA)	
	Crystallization	Crystals	Mother Liquors
Batch #1	1	2.83×10^3	2.68×10^3
	2	2.84×10^3	2.82×10^3
Solution 1	1	110	105
	2	115	120
Solution 2	1	22	21
	2	23	23
Batch #2	1	3.39×10^3	3.29×10^3
	2	3.50×10^3	3.47×10^3
Batch #3	1	3.08×10^3	2.95×10^3
	2	3.13×10^3	3.19×10^3
Batch #4	1	3.27×10^4	3.14×10^4
	2	3.26×10^4	3.26×10^4
Batch #5	1	3.67×10^5	3.89×10^5
	2	3.68×10^5	3.67×10^5

*All solutions were standardized against deoxycorticosterone. The acetic anhydride of Batches #1, #2 and #3 was labeled with ^{14}C while that of Batches #4 and #5 was labeled with ^{3}H.

activities, one of the two following methods were used:

a) When isolated in sufficient amounts the metabolites were crystallized directly from an appropriate solvent mixture until the final specific activities of the crystals and mother liquor did not differ by more than 10%. Following each crystallization a 0.400 mg to 1 mg aliquot of the crystals was removed and weighed accurately on a microbalance (Mettler Model

1-912-3 x 3) before being transferred to counting vials or to volumetric flasks from which aliquots were taken for counting. Mother liquors from crystallizations were transferred to small (2.0-3.0 g) thin-glass Erlenmeyer flasks which were accurately weighed. After drying and re-weighing, the contents of the flasks were quantitatively transferred to volumetric flasks and suitable aliquots were counted. The balance used was accurate to 10 μg in the weight range of the weighing paper and flasks. In the case of certain metabolites, as little as 110 μg was available for weighing. At this level, errors in weighing were about 5%. Sufficient counts were accumulated to give a standard deviation of not more than 2%.

b) In most instances the isolated urinary steroid did not have sufficient weight to determine its specific activity by direct crystallization. The specific activity of these steroids was determined by acetylation with labeled acetic anhydride using the isotope derivative principle described by Kliman and Peterson (43). The isolated compound containing ^{3}H was acetylated with one of the standardized solutions of (1-^{14}C)-acetic anhydride. Those compounds containing ^{14}C were acetylated with one of the ^{3}H-acetic anhydride solutions. The product was mixed with the appropriate carrier acetate and after chromatography of the mixture on a small alumina column, it was crystallized until the $^{3}H/^{14}C$ ratios and the specific activities of the crystals and mother liquors were constant. The specific activity of the isolated steroid was calculated according to the

expressions:

$$S.A. = \frac{axbxdxn}{C}, \text{ when } 1\text{-}^{14}C\text{-acetic anhydride was used,}$$

$$\text{and } S.A. = \frac{axbxn}{dxC}, \text{ when } ^{3}H\text{-acetic anhydride was used.}$$

where S.A. = specific activity of the isolated metabolite

a = specific activity of DOCA (dpm ^{14}C or $^{3}H/\mu g$)

b = molecular weight of DOCA

C = molecular weight of the metabolite

d = $^{3}H/^{14}C$ ratio of the acetylated metabolite

n = number of acylable groups.

Determination of Weights of the Isolated Urinary Steroids

The isolation of minute amounts of 15α-hydroxylated steroids from urine was greatly facilitated by the addition of a small amount of the labeled steroid of known specific activity into urine pools. Following hydrolysis of the urinary steroid conjugates and purification of the extract by various chromatographic procedures, the purified fraction containing the radioactivity was acetylated with acetic anhydride having a different label and the product mixed with the appropriate carrier acetate. After chromatography of the mixture on a small alumina column, it was crystallized until the $^{3}H/^{14}C$ ratios of the crystals and mother liquors were constant and the specific activity of the isolated steroid was calculated as previously described. Then the weight of the steroid originally present in the urine can be calculated according to the well known isotope dilution

equation:

$$A = \frac{sa_1}{sa_2} - 1 \quad x \; w$$

where A = weight of the isolated steroid

sa_1 = specific activity of the steroid added in urine

sa_2 = specific activity of the isolated steroid

and w = weight of the steroid added in urine.

Determination of the Percent Conversion of Injected Steroids to Urinary Metabolites

The percent conversion of an injected steroid to its urinary metabolites was calculated using one of two methods. Where the endogenous specific activity of a metabolite was obtained by direct crystallization of the steroid, the specific activity and the weight of the pure crystals were used to calculate the percent conversion of the injected dose to the urinary metabolite. When the isolated metabolite could not be obtained in a crystalline form it was acetylated with labeled acetic anhydride and the product mixed with the appropriate carrier acetate. The mixture was chromatographed on a small alumina column and crystallized to constant specific activity. This specific activity and the weight of carrier acetate were used to calculate the percent conversion of the injected dose to the urinary metabolite.

Preparation of Labeled Substrates

Labeled 15α-hydroxylated steroids were prepared by the incubation of appropriate labeled substrates with either a strain of Penicillium (ATCC 11598) or with the organism, Colletotrichum linicola, kindly supplied by Dr. P.A. Diassi, the Squibb Institute for Medical Research, New Brunswick, New Jersey. These organisms are capable of hydroxylating the steroid nucleus at the 15α-position (2,4).

(7-^{3}H)-15α-Hydroxyprogesterone. This substrate was prepared by the incubation of (7-^{3}H)-progesterone with Colletotrichum linicola. The microbiological hydroxylation was performed through the courtesy of Dr. P.A. Diassi. Colletotrichum linicola was maintained on slants of Gould's agar. The spores from a one to two-week growth were transferred to a 250 ml Erlenmeyer flask containing 50 ml of sterile medium which consisted of 10 g of brown sugar, 30 g of Cornsteep liquors, 2 g of lard oil, 6 g of sodium nitrate, 5 g of calcium carbonate, 1.5 g of monobasic potassium phosphate, 0.001 g of zinc sulfate and 0.5 g of magnesium sulfate, all in one liter of distilled water. The inoculated medium was incubated at 25°C and 180 cycles per min in a Gyrotory Shaker (New Brunswick Scientific Co., New Jersey) in order to initiate and maintain growth of the microorganism. After 46 hours of shaking a 10% transfer of the growing organism was made to each of ten 250 Erlenmeyer flasks containing 50 ml of sterile medium, pH 7.0, which consisted of 6 g of cornsteep liquors, 2.2 g of soybean

oil, 3 g of ammonium phosphate and 2.5 g of calcium carbonate, all in one liter of distilled water. Tritium labeled progesterone was added in 2 x 0.2 ml of acetone to the flasks, and the mixture incubated for 70 hours as before.

The substrate, 1 mC of (7-^{3}H)-progesterone in 0.031 mg was used as purchased. Prior to use, it was diluted with 100 mg of carrier to give a theoretical specific activity of 2.22×10^7 dpm/mg. Ten mg of the diluted (7-^{3}H)-progesterone was added to each of the ten flasks.

After incubation, the contents of the flasks were pooled and the medium was adjusted to pH 4.0 with sulfuric acid. It was filtered through a large sintered glass funnel and the filtrate (630 ml) was extracted with 4 x 400 ml methylene chloride. After evaporation of the methylene chloride a residue was obtained which weighed 67.5 mg and contained 8.72×10^8 dpm. This residue was crystallized from acetone-hexane to give 27.5 mg of crystals: mp 210-220°C, specific activity 1.68×10^7 dpm/mg. Preliminary chromatographic analysis on paper in systems A and H indicated that the crystals were contaminated with small amounts (maximum 5%) of 12β,15α-dihydroxyprogesterone, 11α,15α-dihydroxyprogesterone and progesterone. An aliquot of the crystals (10.2mg and 1.71×10^8 dpm) was chromatographed on four thin layer plates in system N. The ultraviolet-absorbing zone corresponding in mobility to 15α-hydroxyprogesterone was eluted to give a residue which weighed 8.5 mg and contained 1.45×10^8 dpm. Crystallization from acetone yielded 5.6 mg of small plates:

mp 230-232°C, specific activity 1.74×10^7 dpm/mg. The mother liquor had a specific activity of 1.70×10^7 dpm/mg. The infrared spectrum (KBr) of the crystals was identical to that of 15α-hydroxyprogesterone. These crystals were used in Experiments A-1 and C-1 to be described. The yield of (7-^{3}H)-15α-hydroxyprogesterone was 23%.

An aliquot of the crystalline material containing 1.25×10^5 dpm was mixed with 31.8 mg of carrier steroid and the mixture crystallized as shown in Table 3.

TABLE 3

Proof-of-Radiochemical Purity of (7-^{3}H)-15α-Hydroxyprogesterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	3900	4200
2	4100	4100
3	4050	4000
Calculated*	4000	

*A total of 1.25×10^5 dpm was mixed with 31.8 mg of carrier 15α-hydroxyprogesterone prior to crystallization.

(4-^{14}C)-15α-Hydroxyprogesterone. Carbon-14 labeled 15α-hydroxyprogesterone was prepared from 200 μC of (4-^{14}C)-progesterone (specific activity 57.3 mC/mM, 1.10 mg, 4.50×10^8 dpm). The purity of (4-^{14}C)-progesterone was proved by reverse isotope dilution analysis of an aliquot as shown in Table 4. The remaining labeled steroid was incubated with Penicillium (ATCC

TABLE 4

Proof of Radiochemical Purity of (4-^{14}C)-Progesterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	1390	1470
2	1410	1430
Calculated*	1440	

*A total of 40,800 dpm was mixed with 28.3 mg of carrier prior to crystallization.

11598) using identical conditions to those described for the hydroxylation of (7-^{3}H)-progesterone with Colletotrichum linicola.

The methylene chloride extract obtained after incubation weighed 9.5 mg and contained 4.07×10^{8} dpm. It was chromatographed on paper in system A for 8 hours, and the area corresponding in mobility to 15α-hydroxyprogesterone was eluted to yield 2.8 mg of residue containing 1.05×10^{8} dpm. Chromatography on paper in system C for 5 hours gave one radioactive zone with the mobility of 15α-hydroxyprogesterone. Elution of this zone with methanol afforded a residue which contained 9.00×10^{7} dpm. It was chromatographed on a 1 g alumina column and elution with 1.5% ethanol in benzene yielded a residue containing 8.90×10^{7} dpm. An aliquot of this material was mixed with carrier and crystallized to constant specific activity as shown in Table 5. The yield of (4-^{14}C)-15α-hydroxyprogesterone was 20%.

Another aliquot of (4-^{14}C)-15α-hydroxyprogesterone con-

TABLE 5

Proof of Radiochemical Purity of (4-^{14}C)-15α-Hydroxyprogesterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	2250	2220
2	2180	2140
3	2200	2240
Calculated*	2330	

*A total of 1.12×10^5 dpm was mixed with 48.0 mg carrier prior to crystallization.

taining 1.20×10^5 dpm was acetylated with ^{3}H-acetic anhydride Batch No. 5 and the product was mixed with 65.0 mg of carrier 15α-acetoxyprogesterone. The mixture was chromatographed on a 7 g alumina column and the material eluted with benzene-Skellysolve B (4:1) was crystallized to constant ^{3}H/^{14}C ratio as shown in Table 6. The third crystals and mother liquors were combined to give 32.5 mg of material. It was dissolved in 8.8 ml of methanol and 7.5 mg of $NaBH_4$ was added to the solution. When the reaction was complete, the product was extracted with ethyl acetate. After evaporation of the solvent the residue obtained was dissolved in 2.5 ml of dioxane to which was added 27.5 mg of DDQ reagent. Following oxidation the product formed was purified by chromatography on a 4 g alumina column, and elution with benzene yielded 22.8 mg of material containing 3.9×10^4 dpm of ^{3}H and 3.4×10^4 dpm of ^{14}C. This derivative was crystallized from ether-methanol, and from methanol-ether-

TABLE 6

Determination of the Specific Activity of (4-^{14}C)-15α-Hydroxyprogesterone

Crystallization	Crystals	Specific Activity (dpm ^{3}H/mg) ^{3}H/^{14}C	Mother Liquor	^{3}H/^{14}C
15α-Acetoxyprogesterone*				
1	1940	1.30	7600	4.90
2	1680	1.14	2580	1.70
3	1700	1.13	1730	1.14
15α-Acetoxy-20β-hydroxypregn-4-en-3-one				
1	1720	1.13	1750	1.14
2	1690	1.13	1710	1.13
Calculated**	1710			

*An aliquot of (4-^{14}C)-15α-hydroxyprogesterone containing 1.20x 10^{5} dpm was acetylated with ^{3}H-acetic anhydride Batch No. 5, and the product mixed with 65.0 mg of carrier 15α-acetoxyprogesterone. The mixture was chromatographed on a 7 g alumina column prior to crystallization.

**The third crystals and mother liquors were combined (32.5 mg) and the mixture reduced with $NaBH_4$ and subsequently oxidized with DDQ. The product was then chromatographed on a small alumina column to yield 21.0 mg of material containing 3.59x 10^{4} dpm of ^{3}H and 3.18x10^{4} dpm of ^{14}C.

Skellysolve B, and constant ^{3}H/^{14}C ratios were obtained as shown in Table 6. From the final ^{3}H/^{14}C ratio, the specific activity of (4-^{14}C)-15α-hydroxyprogesterone was calculated to be 3.66x10^{8} dpm/mg.

(4-^{14}C)-15α-Hydroxyandrostenedione. When purchased, 200 μC of (4-^{14}C)-androstenedione had a specific activity of 58 mC/mM and contained 0.99 mg and 4.53x10^{8} dpm. An aliquot

of this material was checked for purity by reverse isotope dilution as shown in Table 7. The remaining labeled steroid

TABLE 7

Proof of Radiochemical Purity of (4-^{14}C)-Androstenedione

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	1360	1280
2	1320	1340
Calcualted*	1310	

*A total of 3.20×10^4 dpm was mixed with 24.5 mg of carrier and crystallized.

was incubated with Penicillium using identical conditions as described for the 15α-hydroxylation of progesterone.

The residue of the methylene chloride extract obtained after extraction of the incubation mixture (200 mg, 4.22×10^8 dpm) was chromatographed on two papers in system L for 7 hours. The UV positive area corresponding to 15α-hydroxyandrostenedione standard was eluted to yield 9.3 mg of residue which contained 2.17×10^8 dpm. This eluate was chromatographed on paper in system J for 4 hours and the radioactive zone with a mobility of 9.5 cm, corresponding to standard, was eluted to yield 2.0 mg of residue containing 1.88×10^8 dpm. The yield of (4-^{14}C)-15α-hydroxyandrostenedione at this stage was 42%. An aliquot containing 1.42×10^5 dpm was mixed with 21.0 mg carrier and the

mixture was crystallized to constant specific activity as shown in Table 8. Another aliquot of (4-^{14}C)-15α-hydroxyandrostene-

TABLE 8

Proof of Radiochemical Purity of (4-^{14}C)-15α-Hydroxyandrostene-dione

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	7060	6790
2	7100	7000
Calculated*	6760	

*A total of 1.42×10^5 dpm of (4-^{14}C)-15α-hydroxyandrostenedione was mixed with 21.0 mg carrier and crystallized.

dione containing 2.42×10^5 dpm was acetylated with ^{3}H-acetic anhydride Batch no.5, and the product mixed with 65.0 mg of carrier 15α-acetoxyandrostenedione. After chromatography on a 7 g alumina column, 63.0 mg of oily material was obtained by elution with benzene-Skellysolve B (1:1) which could not be crystallized. It was reduced with $NaBH_4$ and then oxidized with DDQ as previously described to yield 59.0 mg of material which was chromatographed on a 6 g alumina column. Elution with 0.5% ethanol in benzene gave 41.0 mg of material which was crystallized to constant $^3H/^{14}C$ ratio as shown in Table 9. The third crystals and mother liquors were combined (18.0 mg) and the mixture was acetylated. The resulting acetate was

TABLE 9

Determination of the Specific Activity of (4-^{14}C)-15α-Hydroxy-androstenedione

Crystallization	Specific Activity (dpm ^{3}H/mg) Crystals	^{3}H/^{14}C	Mother Liquor	^{3}H/^{14}C
15α-Acetoxytestosterone*				
1	4570	1.55	54000	18.00
2	3320	1.10	8400	2.80
3	3230	1.06	3140	1.05
15α-Hydroxytestosterone Diacetate				
1	2900	1.06	2920	1.05
2	2860	1.06	2900	1.06
Calculated**	2880			

* An aliquot of (4-^{14}C)-15α-hydroxyandrostenedione was acetylated with ^{3}H-acetic anhydride Batch No.5. The product was mixed with 65.0 mg of carrier 15α-acetoxyandrostenedione and the mixture reduced with $NaBH_4$ and then oxidized with DDQ. The resulting 15α-acetoxytestosterone was chromatographed on alumina prior to crystallization.

**The third crystals and mother liquors were used for the preparation of this derivative. The calculated specific activity was computed from the final specific activity of 15α-acetoxytestosterone and the altered molecular weight.

chromatographed on a 3 g alumina column and elution with benzene-Skellysolve B (1:1) yielded 19.0 mg of material which was crystallized to constant ^{3}H/^{14}C ratio as shown in Table 9. From the final ^{3}H/^{14}C ratio the specific activity of (4-^{14}C)-15α-hydroxyandrostenedione was calculated to be 4.26×10^{8} dpm/mg.

(4-^{14}C)-15α-Hydroxytestosterone. An aliquot of (4-^{14}C)-15α-hydroxyandrostenedione containing 1.45x10^{7} dpm was reduced with $NaBH_4$ and the product oxidized with DDQ as previously described. After oxidation, the mixture was chromatographed on paper in system L for 8 hours and the radioactive zone with the mobility of 15α-hydroxytestosterone was eluted to give a residue containing 9.50x10^{6} dpm. Chromatography on paper in system J for 10 hours gave a single peak of radioactivity corresponding in mobility to 15α-hydroxytestosterone. The eluate from the paper contained 8.80x10^{6} dpm. The yield of (4-^{14}C)-15α-hydroxytestosterone was 61%. An aliquot of this material containing 8.25x10^{4} dpm was mixed with 40.0 mg of carrier and crystallized to constant specific activity as shown in Table 10. The calculated specific activity of (4-^{14})-15α-hydroxy-

TABLE 10

Proof of Radiochemical Purity of (4-^{14}C)-15α-Hydroxytestosterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	2000	2100
2	2050	2080
Calculated*	2060	

*A total of 8.25x10^{4} dpm of (4-^{14}C)-15α-Hydroxytestosterone was mixed with 40.0 mg carrier and crystallized.

testosterone based on the specific activity of (4-^{14}C)-15α-hydroxyandro

tenedione and the increase in molecular weight was 4.23×10^8 dpm/mg.

Purity of Labeled Substrates

Tritium and carbon-14 labeled progesterone was purchased from the New England Nuclear Corporation. On arrival, they were stored in benzene:methanol (3:2). Aliquots of these solutions were further diluted with methanol for counting.

To test the radiochemical purity of (7-^{3}H)-progesterone, a total of 2.18×10^5 dpm was mixed with 48.4 mg of carrier and the mixture was crystallized to constant specific activity as shown in Table 11. From these data it was calculated that the

TABLE 11

Proof of Radiochemical Purity of (7-^{3}H)-Progesterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	4380	4650
2	4430	4540
Calculated*	4510	

*A total of 2.18×10^5 dpm of (7-^{3}H)-progesterone was mixed with 48.4 mg of carrier and crystallized.

(7-^{3}H)-progesterone was as least 97% pure. The specific activity of (7-^{3}H)-progesterone was 200 mC/mM.

The radiochemical purity of (4-^{14}C)-progesterone (specific activity 57.3 mC/mM) was checked by reverse isotope dilu-

tion as shown in Table 12. From these data it was calculated

TABLE 12

Proof of Radiochemical Purity of (4-^{14}C)-Progesterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	750	790
2	740	760
Calculated*	780	

*A total of 3.35×10^4 dpm of (4-^{14}C)-progesterone was mixed with 42.8 mg of carrier and crystallized.

that the (4-^{14}C)-progesterone was at least 98 per cent pure.

Preparation of Non-Labeled 15α-Hydroxyprogesterone

When these studies were started 1.0 g of non-labeled 15α-hydroxyprogesterone was generously donated by Dr. P.A. Diassi, the Squibb Institute for Medical Research, New Brunswick, New Jersey. Because larger amounts of 15α-hydroxyprogesterone were needed, it was prepared by incubating 40 g of progesterone with Penicillium (ATCC 11598). The incubation conditions used were the same as those described for the 15α-hydroxylation of (7-^{3}H)-progesterone except that a large fermenter was used for the incubation of 40 g of progesterone using a concentration of 400 mg of progesterone per liter of incubation medium. The incubation and extraction steps were done through the courtesy of Dr. C. Vezina, Ayerst Laboratories, Montreal.

The final methylene chloride extract weighed 11.2 mg and it was chromatographed on 860 thin layer plates using system N. The ultraviolet-absorbing band corresponding in mobility to 15α-hydroxyprogesterone (average R_f = 0.46) was eluted from the plates to yield 8.12 g of material which was crystallized twice from acetone to give 6.45 g of coarse needles: mp 230-232°C, mmp with standard 15α-hydroxyprogesterone 231-232°C; mp of authentic 15α-hydroxyprogesterone 231-232°C. Its infrared spectrum (KBr) was identical to that of 15α-hydroxyprogesterone. When small aliquots of the crystals were chromatographed on a thin layer plate in system N, only one ultraviolet-absorbing spot with the mobility of 15α-hydroxyprogesterone was obtained.

Non-labeled 15α-acetoxyprogesterone to be used as carrier in the studies to be described was prepared by the acetylation of 1.50 g of 15α-hydroxyprogesterone. The product was chromatographed on a 200 g alumina column from which 1.58 g of crystalline material was eluted with 80% benzene in Skellysolve B. Crystallization from acetone-Skellysolve B afforded 1.30 g of plates: mp 179-181°C. Comparison of the infrared spectrum (CS_2) of this material with that of 15α-hydroxyprogesterone showed an absence of the hydroxyl band and the appearance of acetate bands at 1245 and 1738 cm^{-1}.

Preparation of 15α-Acetoxytestosterone and 15α-Hydroxytestosterone Diacetate

Non-labeled 15α-hydroxyandrostenedione and 15α-hydroxy-

testosterone were kindly supplied by Dr. P.A. Diassi. Acetylation of 100 mg of 15α-hydroxytestosterone and chromatography of the product on a 12 g alumina column yielded 130 mg of oily material on elution with 40% benzene in Skellysolve B. Crystallization from acetone-Skellysolve B afforded 83.0 mg of crystalline 15α-hydroxytestosterone diacetate: mp 154-156°C. Its infrared spectrum (CS_2) showed an absence of the hydroxyl band and the appearance of acetate bands at 1235 and 1740 cm^{-1}.

Acetylation of 200 mg of 15α-hydroxyandrostenedione gave a product which was chromatographed on a 25 g alumina column. Elution with 50% benzene in Skellysolve B yielded 212 mg of oily material which could not be crystallized. It was reduced with $NaBH_4$, oxidized with DDQ, and the product chromatographed on a 20 g alumina column. Elution with 0.5% ethanol in benzene afforded 142 mg of material which was crystallized from acetone to yield 112 mg of fine needles: mp 227-230°C. Its infrared spectrum (KBr) showed the presence of the Δ^4-3-ketone and acetate bands at 1711, 1270 and 1256 cm^{-1}. Acetylation of an aliquot of this material gave a product which had an infrared spectrum (CS_2) identical to that of 15α-hydroxytestosterone diacetate prepared by acetylation of 15α-hydroxytestosterone as described above. Therefore, the material prepared was identified as 15α-acetoxytestosterone.

EXPERIMENTAL SECTION AND RESULTS

SECTION A. ISOLATION OF 15α-HYDROXYLATED NEUTRAL STEROIDS FROM HUMAN URINE

Experiment A-1 Isolation of 15α-Hydroxyprogesterone from Human Urine

This initial study was designed to isolate a sufficient amount of crystalline 15α-hydroxyprogesterone from urine in order to identify it by its infrared spectrum, mp and mixed mp.

A 14-day urine collection was obtained from a normal subject in the 35th week of gestation and 5.50×10^5 dpm of purified (7-^{3}H)-15α-hydroxyprogesterone, specific activity 1.74×10^4 dpm/μg, was added to the urine. The urinary conjugates were hydrolyzed with Glusulase as previously described, and the neutral extract obtained weighed 3.5 g and contained 5.20×10^5 dpm. It was chromatographed on a 350 g silica gel column which was developed first with methylene chloride and then with increasing concentrations of ethanol in methylene chloride. The effluent was collected in 200 ml fractions at a rate of one fraction per two hours and a single radioactive band was eluted with 3% ethanol in methylene chloride (fractions 38-47). The residue obtained from this peak weighed 182 mg and contained 4.26×10^5 dpm. It was further purified by chromatography on a 20 g alumina column and elution with 1.5% ethanol in

benzene gave 42 mg of material which contained 4.00×10^5 dpm. This material was chromatographed on two sheets of Whatman paper, No. 3MM, in system A for 10 hours. The radioactive material eluted with the mobility of 15α-hydroxyprogesterone weighed 11 mg and contained 3.90×10^5 dpm. It was further purified by chromatography on paper in system B for 5 days. Two major ultraviolet-absorbing bands were observed corresponding in mobility to 15α- and 16α-hydroxyprogesterone. The radioactive band on the paper corresponded in mobility to 15α-hydroxyprogesterone and it was eluted to give a residue which weighed 1.4 mg and contained 3.40×10^5 dpm. Further purification of this material was achieved by chromatography on a 1 g alumina column, and elution with 1.5% ethanol in benzene gave an oil which weighed 0.58 mg and contained 3.00×10^5 dpm. Crystallization of this material from acetone-ether-Skellysolve B afforded 0.11 mg of small plates containing 1.38×10^5 dpm, mp 230.5-232°, mmp 230-232°; authentic 15α-hydroxyprogesterone, mp 231-232°. The infrared spectrum (KBr) of this material was identical to that of authentic 15α-hydroxyprogesterone as shown in Figure 1. The isolated 15α-hydroxyprogesterone had a specific activity of 1.25×10^3 dpm/μg.

An aliquot of the crystals containing 6.70×10^4 dpm was acetylated with (1-^{14}C)-acetic anhydride, Batch No. 1. The product was mixed with 24.3 mg of carrier 15α-acetoxyprogesterone and the mixture was chromatographed on a 3 g alumina column. Elution with benzene-Skellysolve B (4:1) yielded a

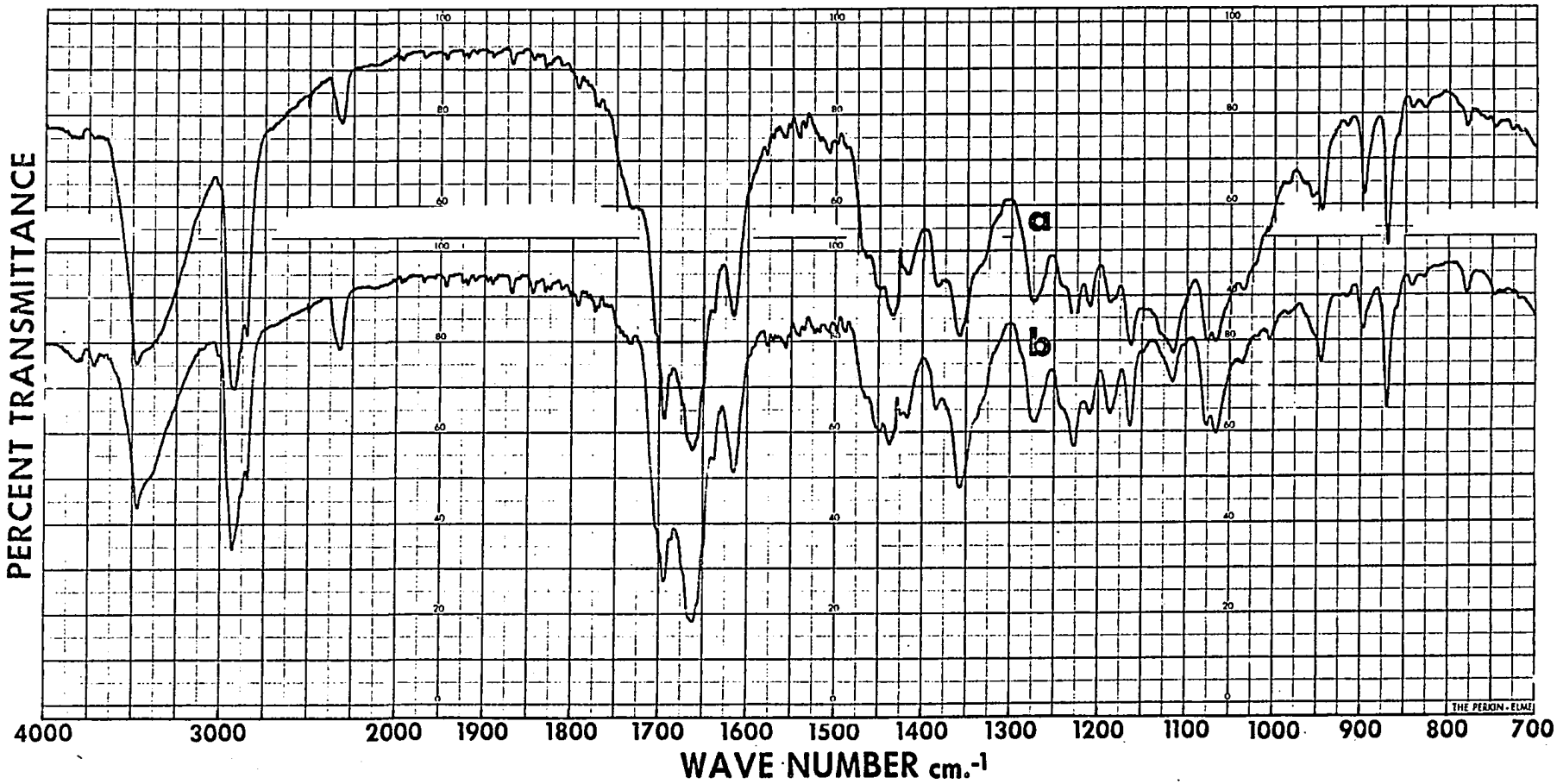


FIG. 1. Infrared spectra (KBr). (a) Isolated urinary steroid.
(b) Authentic 15α-hydroxyprogesterone.

residue which weighed 20.7 mg and contained 5.70×10^4 dpm of ^3H. It was crystallized from methanol-ether, acetone-Skelly-solve B, and ether, and the specific activities as well as the $^3H/^{14}C$ ratios were determined on the crystals and mother liquors, as shown in Table 13. Constant specific activities

TABLE 13

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated From a 14-Day Collection of Human Pregnancy Urine

		Specific Activity (dpm ^{14}C/mg)		
Crystallization	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
15α-Acetoxyprogesterone*				
1	240	11.5	280	9.7
2	240	11.3	250	11.1
3	240	11.2	240	11.4
15α-Acetoxy-20β-hydroxypregn-4-en-3-one**				
1	240	11.2	240	11.3
2	240	11.2	240	11.4
Calculated	240			

* An aliquot of the crystalline 15α-hydroxyprogesterone containing 6.70×10^4 dpm of ^3H was acetylated with ^{14}C-acetic anhydride, Batch No. 1, and the product was mixed with 24.3 mg of carrier 15α-acetoxyprogesterone prior to chromatography of the mixture on alumina and crystallization of the material eluted.

** The third crystals and the second and third mother liquors were combined (13.0 mg), reduced with $NaBH_4$, and the product oxidized with the DDQ reagent and then chromatographed on an alumina column to yield 9.1 mg of material which contained 2.20×10^3 dpm of ^{14}C. The calculated specific activity is based on these values.

were achieved in the crystals and mother liquors. The third crystals as well as the second and third mother liquors were combined to give 13.0 mg of material. It was dissolved in 3.5 ml of methanol and 3.0 mg of $NaBH_4$ was added to the solution. When the reaction was complete, the product was extracted with ethyl acetate. After evaporation of the solvent the residue obtained was dissolved in 1 ml of dioxane to which was added 11 mg of DDQ reagent. Following oxidation the product formed was purified by chromatography on a 2 g alumina column, and elution with benzene yielded 9.1 mg of crystalline material containing 2.50×10^4 dpm of ^3H and 2.20×10^3 dpm of ^{14}C. This derivative was crystallized from ether-methanol, and from methanol-ether-Skellysolve B and the specific activities of the crystals and mother liquors were determined and found to be constant as is shown in Table 13. Using the final $^3H/^{14}C$ ratio and the specific activity of the acetic anhydride, the specific activity of the urinary 15α-hydroxyprogesterone was calculated to be 1.30×10^3 dpm of $^3H/\mu g$. This value was in close agreement with the specific activity of the crystalline 15α-hydroxyprogesterone isolated directly from the urine (1.25×10^3 dpm/μg). From this specific activity it was possible to calculate that the amount of 15α-hydroxyprogesterone excreted in the urine averaged 28 μg/day over a 14-day period.

After having isolated crystalline 15α-hydroxyprogesterone from late pregnancy urine a large number of urine pools was analyzed to determine the concentration of this steroid in the urine of subjects with a variety of endocrine status. The following

subjects were used in these studies:

Subject 1: A normal female in the 35th week of gestation (3rd trimester). The isolation of 15α-hydroxyprogesterone from the urine of this subject was described above.

Subject 2: A normal female in the 34th week of gestation (3rd trimester)

Subject 3: A normal female in the 17th week of gestation (2nd trimester)

Subject 4: A normal female in the 16th week of gestation (2nd trimester)

Subject 5: A normal female in the 9th week of gestation (1st trimester)

Subject 6: A normal female in the 13th week of gestation (1st trimester)

Subject 7: A normal female in the follicular phase of the menstrual cycle

Subject 8: A normal female in the follicular phase of the menstrual cycle

Subject 9: A normal female in the luteal phase of the menstrual cycle.

Subject 10: A normal female in the luteal phase of the menstrual cycle.

Subject 11: A 49 year-old woman with Cushing's Syndrome due to mild adrenal cortical hyperplasia, secondary diabetes mellitus and hypertension. She was grossly obese, the obesity being markedly truncal with relatively thin arms and legs. Her face was round, but not quite a "moon face",

with facial downy hair on the cheeks. The abdomen was grossly obese and pendulous with a lax anterior abdominal wall musculature but with no pink striae. The plasma corticoids were unequivocally increased and a diurnal rhythm in the plasma concentration of cortisol was not observed. The urinary 17-hydroxycorticoids and the 17-ketogenic steroids were elevated about twice the upper range of normal, and the urinary 17-ketosteroids were within the normal range. The most convincing laboratory report for the diagnosis came from the urinary concentration of free cortisol which was several times greater (640-680 µg/day) than normal (120 µg/day). When she was given 8 mg of Dexamethasone daily for three days, there was normal suppression in the plasma and urinary corticoids. Both adrenals were removed through bilateral loin incisions. The right adrenal was about twice the size of the left and the combined weight of the adrenals was 16 g which is at the upper limit of normal. Microscopic sections of the right adrenal revealed thickening of the adrenal cortex involving in particular the zona fasiculata but no evidence of malignancy was obtained. The adrenal cortex was widened. The adrenal medulla was well preserved. Her post-operative course was uncomplicated with reduction in the severity of diabetes mellitus. She was discharged from hospital on 37.5 mg of cortisone acetate and 0.1 mg of 9α-fluorohydrocortisone daily. The urine used for the present study was obtained pre-operatively.

Subject 12: A 37 year-old woman with Cushing's syndrome due to left adrenal cortical adenoma. Physical examination

showed a typical Cushinoid appearance. The face showed the typical "moon face" with a high color to the cheeks. There was drooping of the corner of the mouth and a porcine appearance to the eyes. There were faint, purplish striae on the lower abdominal wall. In addition, there was modest facial hirsutism but no generalized hirsutism and no hypertrophy of the clitoris. A glucose tolerance test showed the presence of diabetes. Examination of the plasma corticoids showed both elevated values for plasma corticoids and reversal of diurnal variation. The urinary 17-hydroxycorticoids were 6 mg/24 hrs, the 17-ketosteroids were 4 mg/24 hrs, and the ketogenic steroids, 22 mg/24 hrs. Following 2 days suppression with 8 mg Dexamethasone, the 17-hydroxycorticoids rose to 11 mg/24 hrs, the 17-ketosteroids to 9 mg/24 hrs, and the ketogenic steroids to 32 mg/24 hrs. Urinary free cortisol was elevated to 225 µg/24 hrs, and following suppression with 8 mg of Dexamethasone it rose to 450 µg/24 hrs. A left adrenal cortical adenoma was removed through a left flank incision. The post-operative course was essentially uneventful, although the diabetic state was somewhat intensified post-operatively. She was discharged to a convalescent hospital on 37.5 mg of cortisone daily, an 1800 calorie diabetic diet and Phenoformin (DBI-TD) 50 mg daily. The urine used for the present study was obtained pre-operatively.

Subject 13: This 7 year-old white male was a non-salt loser with congenital adrenal hyperplasia with a block of hydroxylation at C-21. The urine from this child was collected in 1956 prior to any treatment and kept constantly frozen

until it was sent to us by Dr. W.R. Eberlein, The Children's Hospital of Philadelphia, in 1967. During the period of urine collection, his 17-ketosteroid excretion varied between 15 and 20 mg/day and his pregnanetriol excretion was 16-25 mg/day. This boy's urine was analyzed before treatment for tetrahydroaldosterone (41 µg/24 hours) and 18-hydroxytetrahydrocorticosterone (380 µg/24 hours). This is one of the group with the abnormal ratio. He has been continuously treated during the past 12 years and has done well.

Urine pool obtained from newborn infants: An 11-day urine pool was obtained from premature infants during the first month of life at the wards of Royal Victoria Hospital, Montreal.

The method of isolation of 15α-hydroxyprogesterone from the urine of these subjects was essentially the same as that described above for the isolation of crystalline 15α-hydroxyprogesterone from the 14-day urine pool obtained from subject 1. The only difference was that the isolated 15α-hydroxyprogesterone was not crystallized, but its concentration in urine was determined by the isotope dilution procedure.

Four to six-day urine collections were usually used for these determinations. To each urine pool was added a known amount of tritium labeled 15α-hydroxyprogesterone of determined specific activity and the urinary steroid conjugates were hydrolyzed with Glusulase or with β-glucuronidase. The specific activity of (7-^{3}H)-15α-hydroxyprogesterone added in the urine was 1.74×10^{4} dpm/µg. The size of the urine collection obtained

from each subject, the amount of (7-^{3}H)-15α-hydroxyprogesterone added to the urine in terms of dpm of ^{3}H and micrograms, and the enzyme used for the hydrolysis of the urinary steroid conjugates are shown in Table 14.

TABLE 14

Urine Collected, Enzyme Used to Hydrolyze Steroid Conjugates and the Weight and Amount of (7-^{3}H)-15α-Hydroxyprogesterone Added to Each Urine Pool

Subject	Urine Collected (days)	Enzyme	(7-^{3}H)-15P* (dpm)	Added in Urine (μg)
2	5	Glusulase	1.74×10^6	100.0
3	4	Glusulase	3.25×10^5	18.6
4	4	Glusulase	3.06×10^5	17.6
5	5	Glusulase	3.09×10^5	17.8
6	5	β-Glucuronidase	1.65×10^5	9.5
7	4	Glusulase	3.28×10^5	18.8
8	6	β-Glucuronidase	1.86×10^5	10.7
9	6	β-Glucuronidase	2.17×10^5	12.5
10	6	β-Glucuronidase	1.75×10^5	10.1
11	4	Glusulase	4.29×10^5	28.3
12	3	Glusulase	3.26×10^5	18.7
13	3	Glusulase	1.35×10^5	7.8
Newborn Infants	11	β-Glucuronidase	1.65×10^5	9.5

*(7-^{3}H)-15P = (7-^{3}H)-15α-Hydroxyprogesterone

Following hydrolysis of the steroid conjugates the neutral extract obtained was purified by chromatography on silica gel and alumina columns, paper chromatography in systems A and B and finally, by chromatography on a small alumina column. At this stage, the residue was acetylated with (1-^{14}C)-acetic anhydride. Acetic anhydride, Solution No. 1, was used for the acetylation of the residues obtained from the urine of Subject 2. The residues obtained from the urine of Subjects 3, 4, 5, 7, 11, 12 and 13 were acetylated using Batch No. 1, and Batch No. 2, was used for the acetylation of the residues obtained from the urine of subjects 6, 8, 9, 10 and the newborn infants. The amounts of radioactivity contained in the samples before acetylation and the quantity of carrier 15α-acetoxyprogesterone added after acetylation are shown in Table 15.

The acetylation mixture containing carrier 15α-acetoxy-progesterone was extracted with ethyl acetate, chromatographed on alumina, and the steroid eluted from the column was crystallized several times until the specific activities of the crystals and the mother liquors and their $^{3}H/^{14}C$ ratios were constant. In each case, the last crystals and mother liquors were combined and the steroid was reduced with $NaBH_4$. Following reduction, the mixture was oxidized with DDQ and the material obtained was purified by chromatography on an alumina column and the residue eluted was crystallized until constant specific activities and $^{3}H/^{14}C$ ratios were obtained in the crystals and mother liquors. The results from the crystallizations establishing the radio-

TABLE 15

Radioactivity Contained in Samples Before Acetylation and Amounts of Carrier 15α-Acetoxyprogesterone Added to the Acetylation Products to Prove Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Urine of Subjects 2-13 and of Newborn Infants

Subject	Radioactivity in Sample before Acetylation (dpm ^3H)	Carrier 15α-Acetoxyprogesterone added after Acetylation (mg)
2	7.70×10^5	52.2
3	9.00×10^4	50.5
4	1.20×10^5	52.0
5	9.80×10^4	30.2
6	8.80×10^4	36.2
7	1.00×10^5	29.5
8	3.20×10^4	29.5
9	8.00×10^4	35.0
10	7.30×10^4	32.0
11	7.70×10^4	31.5
12	9.20×10^4	30.6
13	8.30×10^4	30.9
Newborn Infants	4.50×10^4	31.6

chemical purity of 15α-hydroxyprogesterone isolated from the urine of subjects 2-13 and from the urine of newborn infants are shown in Tables 16-20.

From the final $^3H/^{14}C$ ratio and the specific activity of the acetic anhydride used for acetylation, the specific activity

TABLE 16

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Urine of Subjects 2-4

	Specific Activity (dpm ^{14}C/mg)							
	15α-Acetoxyprogesterone				15α-Acetoxy-20β-hydroxy-pregn-4-en-3-one			
Crystallization	Xls*	$^{3}H/^{14}C$	ML**	$^{3}H/^{14}C$	Xls	$^{3}H/^{14}C$	ML	$^{3}H/^{14}C$
				Subject 2				
1	6770	2.0	15100	0.9	6830	2.0	6800	2.0
2	6660	2.0	7510	1.8	6720	2.0	6700	2.0
3	6680	2.0	6690	2.0				
Calculated***					6750			
				Subject 3				
1	950	1.7	3350	0.5	720	2.3	750	2.3
2	800	2.0	900	1.8	730	2.3	740	2.3
3	740	2.3	800	2.0				
Calculated***					730			
				Subject 4				
1	1440	1.3	12730	0.2	1030	1.9	1010	1.8
2	1020	1.9	3100	0.6	1000	1.9	1020	1.9
3	990	1.9	1080	1.8				
Calculated***					1050			

*Xls = Crystals
**ML = Mother Liquor
***The last crystals and mother liquor were combined, reduced with $NaBH_4$, and the product oxidized with the DDQ reagent and then chromatographed on an alumina column. The calculated specific activities were based on the weight and the ^{14}C content of the material eluted from the alumina column.

TABLE 17

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Urine of Subjects 5 - 7

Crystallization	Specific Activity (dpm ^{14}C/mg)							
	15α-Acetoxyprogesterone				15α-Acetoxy-20β-hydroxy-pregn-4-en-3-one			
	Xls*	$^{3}H/^{14}C$	ML**	$^{3}H/^{14}C$	Xls	$^{3}H/^{14}C$	ML	$^{3}H/^{14}C$
			Subject 5					
1	950	3.3	5980	0.5	750	4.2	760	4.1
2	840	3.7	980	3.2	740	4.2	750	4.2
3	760	4.2	810	3.9				
4	740	4.2	750	4.2				
Calculated***					750			
			Subject 6					
1	1720	1.2	20000	0.1	1130	1.9	1270	1.8
2	1220	1.8	7330	0.3	1160	1.9	1100	1.9
3	1140	1.9	1180	1.9				
Calculated***					1150			
			Subject 7					
1	1030	3.2	1680	1.8	710	4.3	740	4.2
2	730	4.3	1120	2.8	720	4.4	710	4.4
3	710	4.3	760	4.1				
4	720	4.4	720	4.3				
Calculated***					720			

*XLs = Crystals
**ML = Mother Liquor
***The last crystals and mother liquor were combined, reduced with $NaBH_4$, and the product oxidized with the DDQ reagent and then chromatographed on an alumina column. The calculated specific activities were based on the weight and the ^{14}C content of the material eluted from the alumina column.

TABLE 18

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Urine of Subjects 8-10

Crystallization	Specific Activity (dpm ^{14}C/mg)							
	15α-Acetoxyprogesterone				15α-Acetoxy-20β-hydroxypregn-4-en-3-one			
	Xls*	$^{3}H/^{14}C$	ML**	$^{3}H/^{14}C$	Xls	$^{3}H/^{14}C$	ML	$^{3}H/^{14}C$
				Subject 8				
1	230	3.0	1910	0.4	160	4.3	160	4.0
2	170	4.0	580	1.2	160	4.4	170	4.2
3	150	4.4	160	4.3				
Calculated***					160			
				Subject 9				
1	680	3.0	3900	0.5	540	4.0	540	3.8
2	540	3.9	1130	1.9	510	4.0	530	4.0
3	530	4.0	530	3.9				
Calculated***					530			
				Subject 10				
1	700	2.8	20100	0.1	430	4.3	480	4.0
2	470	4.0	1580	1.2	430	4.4	450	4.3
3	450	4.3	470	4.1				
Calculated***					440			

*Xls = Crystals
**ML = Mother Liquor
***The last crystals and mother liquor were combined, reduced with $NaBH_4$, and the product oxidized with the DDQ reagent and then chromatographed on an alumina column. The calculated specific activities were based on the weight and the ^{14}C content of the material eluted from the alumina column.

TABLE 19

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Urine of Subjects 11-13

Crystallization	Specific Activity (dpm ^{14}C/mg)							
	15α-Acetoxyprogesterone				15α-Acetoxy-20β-hydroxypregn-4-en-3-one			
	Xls*	$^{3}H/^{14}C$	ML**	$^{3}H/^{14}C$	Xls	$^{3}H/^{14}C$	ML	$^{3}H/^{14}C$
				Subject 11				
1	630	3.2	1530	1.3	570	3.5	600	3.4
2	610	3.4	820	2.5	580	3.5	580	3.5
3	590	3.5	580	3.5				
Calculated***					570			
				Subject 12				
1	910	3.4	3530	0.9	780	4.0	810	3.9
2	820	3.8	2950	1.1	790	4.0	800	4.0
3	770	4.0	830	3.8				
4	790	4.0	800	3.9				
Calculated***					800			
				Subject 13				
1	640	3.9	5000	0.5	630	4.0	650	3.9
2	620	4.0	700	3.6	620	4.0	630	4.0
3	630	4.0	630	4.0				
Calculated***					630			

*XLs = Crystals
**ML = Mother Liquor
***The last crystals and mother liquor were combined, reduced with $NaBH_4$, and the product oxidized with the DDQ reagent and then chromatographed on an alumina column. The calculated specific activities were based on the weight and the ^{14}C content of the material eluted from the alumina column.

TABLE 20

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Urine of Newborn Infants

	Specific Activity (dpm ^{14}C/mg)							
	15α-Acetoxyprogesterone				15α-Acetoxy-20β-hydroxy-pregn-4-en-3-one			
Crystallization	XLs*	$^{3}H/^{14}C$	ML**	$^{3}H/^{14}C$	XLs	$^{3}H/^{14}C$	ML	$^{3}H/^{14}C$
1	440	1.9	5200	0.2	300	2.9	310	2.6
2	340	2.6	1580	0.5	300	2.9	300	2.8
3	300	2.8	340	2.5				
4	300	2.9	300	2.8				
Calculated***					300			

*XLs = Crystals
** ML = Mother Liquor
***The fourth crystals and mother liquor were combined, reduced with $NaBH_4$, and the product oxidized with the DDQ reagent and then chromatographed on an alumina column. The calculated specific activity was based on the weight and the ^{14}C content of the material eluted from the alumina column.

of the urinary 15α-hydroxyprogesterone was calculated. This final sppecific activity was then used to calculate the amount of 15α-hydroxyprogesterone excreted in the urine. The results obtained from these calculations are shown in Table 21. It is

TABLE 21

Specific Activities and Amounts of 15α-Hydroxyprogesterone Isolated from the Urine of Subjects 1-13 and from the Urine of Newborn Infants

Subject	15α-Hydroxyprogesterone Isolated from Urine Specific Activity (dpm ^{3}H/mg)	Excretion (μg/day)
1	1.30×10^3	28
2	6.40×10^3	34
3	8.60×10^3	5
4	7.30×10^3	6
5	1.58×10^4	0
6	7.30×10^3	3
7	1.63×10^4	0
8	1.57×10^4	0
9	1.43×10^4	0
10	1.57×10^4	0
11	1.30×10^4	2
12	1.50×10^4	1
13	1.45×10^4	0
Newborn Infants	1.09×10^4	0.6

worthwhile to mention here that sizable amounts (200 µg/day) of crystalline 16α-hydroxyprogesterone were isolated from the urine of Subject 13. The infrared spectrum (KBr) of the isolated material was identical to that of authentic 16α-hydroxyprogesterone as shown in Figure 2. The isolated material had a mp of 216-220°C, 16α-hydroxyprogesterone mp 217-220°C, mmp 217-220°C.

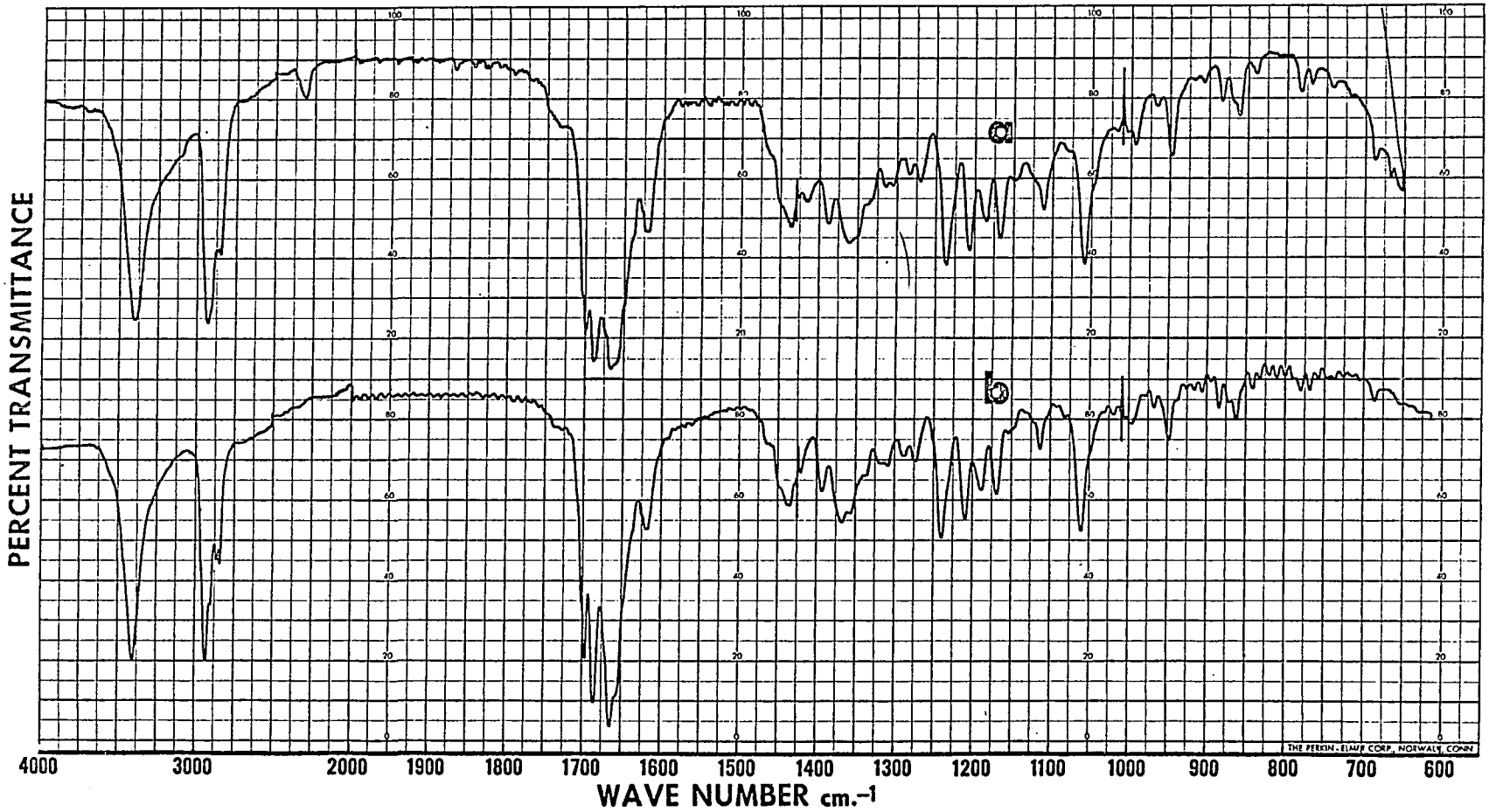


FIG. 2. Infrared Spectra (KBr). (a) Steroid isolated from the urine of Subject 13. (b) Authentic 16α-Hydroxyprogesterone.

Experiment A-2. Isolation of 15α-Hydroxyandrostenedione and 15α-Hydroxytestosterone from Human Late Pregnancy Urine.

A 14-day urine collection was obtained from a normal subject in the 36th to 38th week of gestation and 5.40×10^5 dpm of purified (4-^{14}C)-15α-hydroxyandrostenedione, specific activity 4.26×10^5 dpm/μg, was added to the urine. The urinary conjugates were hydrolyzed with β-glucuronidase and the neutral extract obtained weighed 5.7 g and contained 4.10×10^5 dpm of ^{14}C. It was chromatographed on a 300 g silica gel column which was developed with methylene chloride and then with increasing concentrations of ethanol in methylene chloride. The effluent from the column was collected at the rate of 80-90 ml/hour, in 20 ml fractions. A single peak of radioactive material was eluted with 3% ethanol in methylene chloride (fractions 360-440). The residue obtained from this peak weighed 162 mg and contained 3.66×10^5 dpm. It was further purified by chromatography on a 15 g alumina column and elution with 1.5% ethanol in benzene gave 58 mg of material which contained 3.32×10^5 dpm; This material was chromatographed on two sheets of Whatman paper, No. 3MM, in system A for 10 hours. Two major ultraviolet-absorbing bands were observed, the more polar of the two having the mobility of 15α-hydroxyandrostenedione and the less polar band corresponding in mobility to 15α- and 16α-hydroxyprogesterone. On scanning the paper for radioactivity, one radioactive peak was obtained corresponding to the more polar

ultraviolet-absorbing band which had the mobility of 15α-hydroxyandrostenedione. This material was eluted from the paper to yield 12 mg of residue containing 3.20×10^5 dpm which was further chromatographed on paper in system C for 6 hours. Two ultraviolet-absorbing bands were obtained on this chromatogram with average mobilities of 12 and 16 cm from the origin. The more polar band contained radioactive material and had the mobility of 15α-hydroxyandrostenedione. The less polar band was devoid of radioactivity and attempts to identify this material will be described later.

The material having the mobility of 15α-hydroxyandrostenedione was eluted from the paper to yield a residue which weighed 2.9 mg and contained 2.81×10^5 dpm. It was chromatographed on paper in system G for 55 hours and three ultraviolet-absorbing bands were obtained with average mobilities of 3, 17 and 21 cm from the origin. The band at 17 cm having the mobility of 15α-hydroxyandrostenedione and containing radioactive material was eluted to yield a residue which weighed 4.0 mg and contained 2.20×10^5 dpm. Following chromatography of this material on a 1 g alumina column it was acetylated with ^3H-acetic anhydride, Batch No. 4, and the resulting acetate was extracted to yield a residue containing 1.50×10^5 dpm of ^{14}C and 6.40×10^6 dpm of ^3H. Chromatography of the acetate on paper in system D for 10 hours and scanning of the paper gave one peak of radioactive material containing 8.70×10^4 dpm of ^{14}C and 2.40×10^6 dpm of ^3H as well as six additional peaks

of radioactivity containing only ^{3}H. The $^{3}H/^{14}C$ ratio of the material eluted from the radioactive peak containing both labels was 27.6. On successive chromatography of the material on paper in systems B and G, the $^{3}H/^{14}C$ ratio was found to be 14.2 and 9.0, respectively.

To the residue eluted from the last paper containing $1.50x10^{5}$ dpm of ^{3}H and $1.66x10^{4}$ dpm of ^{14}C, 5.0 mg of carrier 15α-acetoxyandrostenedione was added and the mixture was reduced with $NaBH_4$ and then oxidized with the DDQ reagent. The residue obtained after extraction of the oxidation mixture was mixed with 34.2 mg of carrier 15α-acetoxytestosterone. This material was chromatographed on a 4 g alumina column and elution with 0.5% ethanol in benzene yielded 40.0 mg of crystalline material containing $7.32x10^{4}$ dpm of ^{3}H and $7.65x10^{3}$ dpm of ^{14}C. It was crystallized from acetone, methanol, and acetone-Skellysolve B and constant specific activities and $^{3}H/^{14}C$ ratios were obtained as shown in Table 22. The third crystals and mother liquor were combined (12.0 mg) and acetylated. The diacetate was chromatographed on a 2 g alumina column and elution with benzene-Skellysolve B (1:1) gave an oil (13.2 mg and $1.20x10^{4}$ dpm of ^{3}H) which was crystallized to constant specific activity and constant $^{3}H/^{14}C$ ratio as shown in Table 22. From the final $^{3}H/^{14}C$ ratios and the specific activity of the acetic anhydride, the specific activity of the urinary 15α-hydroxyandrostenedione was calculated to be $7.31x10^{3}$ dpm of $^{14}C/\mu g$. Using this value it was calculated that the amount of 15α-hydroxyandrostenedione excreted in the urine was 5 μg/day.

TABLE 22

Proof of Radiochemical Purity of 15α-Hydroxyandrostenedione Isolated from a 14-Day Collection of Pregnancy Urine

Crystallization	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
15α-Acetoxytestosterone*				
1	1220	6.6	4510	23.7
2	1020	5.6	1750	9.8
3	1030	5.5	1010	5.5
15α,17β-Diacetoxy-androst-4-en-3-one				
1	900	5.6	910	5.7
2	910	5.5	900	5.6
Calculated**	910			

*To the residue obtained after oxidation with the DDQ reagent 34.2 mg of carrier 15α-acetoxytestosterone was added prior to chromatography of the mixture on alumina and crystallization of the material eluted.

**The third crystals and mother liquor were combined (12.0 mg) and acetylated. The diacetate was chromatographed on an alumina column to give an oil which weighed 13.2 mg and contained 1.20×10^4 dpm of ^3H. The calculated specific activity was based on these values.

On page 71 it was mentioned that an ultraviolet-absorbing band was observed on the paper chromatogram having a mobility greater than that of 15α-hydroxyandrostenedione in system C. Following elution of this material from the paper and chromatography on a small alumina column, a crystalline material was obtained which weighed 0.90 mg. The infrared spectrum (KBr) of a 30 μg aliquot is shown in Figure 3. This spectrum showed absorption bands at 1660 cm^{-1} (α,β unsaturated ketone), 1610

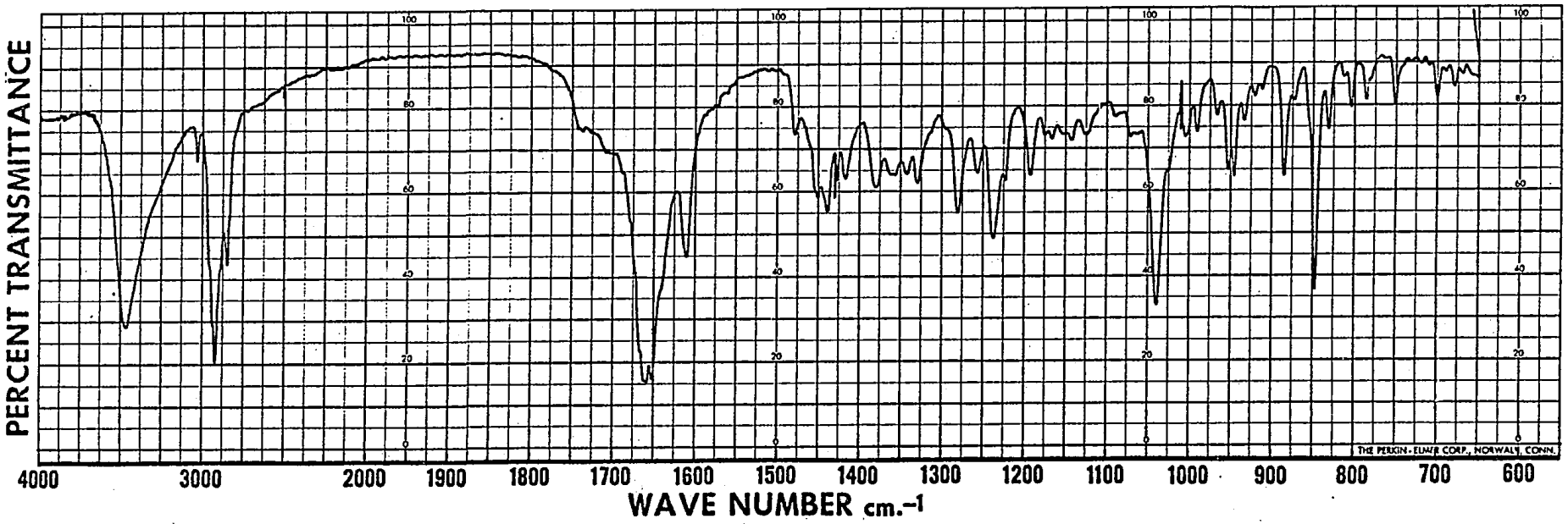


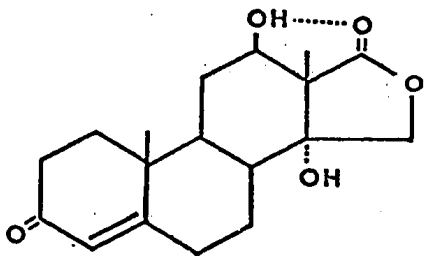
FIG. 3. Infrared Spectrum (KBr) of unknown steroid isolated from pregnancy urine.

cm^{-1} (C=C) and 3465 cm^{-1} (OH stretching). There was no 1740 cm^{-1} band for a 17-ketone or 1710 cm^{-1} band for a 20-ketone. The mass spectrum showed highest m/e at 302. The 100 Mc NMR spectrum (courtesy of Dr. G. Slomp, The Upjohn Company) using the Varian HA-100 spectrometer with the C01024 Time Averaging Computer, showed the general characteristics of steroid NMR spectra. The spectrum showed a singlet absorption at 5.73 ppm attributed to the 4-hydrogen of a 3-keto-Δ^4-steroid. The unusual 3-keto-Δ^4-fingerprint was observed at 2.35 ppm attributed to the hydrogens at C-2 and C-6. There were two singlet absorptions in the methyl-hydrogen absorption region. The least shielded one, at 1.27 ppm was attributed to a 10-methyl and the more shielded one, at 1.19 ppm, to a 13-methyl. The appearance of 18-hydrogen signals this high is often associated with an unusual D-ring; e.g., 14-isosteroids, D-homosteroids or perhydrophenanthrene without a D-ring closure, 15β-hydroxy, 16-, 17-epoxy or 17-ketosteroids. The spectrum showed a broad singlet at 1.60 ppm which was attributed to at least two hydroxyl groups. The spectrum also showed an unusual carbinol-hydrogen region which changed as the chloroform solution aged. Early spectra contained an AB multiplet, $A = 3.87$, $B = 3.41$, $AB = 11.0$ and an S multiplet of an ABX system, $X = 3.48$, $AX = 3.0$, $BX = 12.0$ and after three days in the spectrometer the spectrum showed several new absorptions in the carbinol region. At equilibrium, the B part of the AB multiplet shifted to 3.62 ppm and the X multiplet became a singlet at 3.77 ppm. Other new ab-

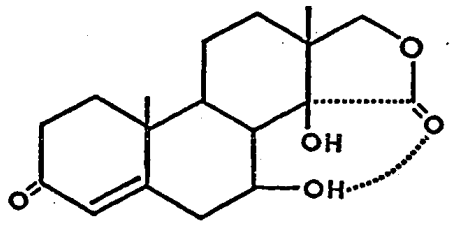
sorptions appeared at 4.0, 4.3 and 4.5 ppm.

The presence of a Δ^4-3-ketone appears certain from the NMR data and the infrared spectrum. In addition, the molecule must contain two hydroxyl groups, CH_2O and H-C-O functions. Reasonable structures with m/e 302 which contain at least the ABC rings of a 3-keto-Δ^4-steroid and at least two oxygens are shown in Figure 4. Structure I would fit best the NMR data (see Table 23). The molecule would be expected to dehydrate easily in the mass spectrometer to furnish a m/e 302 ion. The hydrogen bonding should increase the carbonyl absorption frequency in the direction of 1660 cm^{-1} which could account for the lack of two carbonyl bands in the infrared spectrum. The substituent contributions of the 16-oxygen of the singular methyl absorption in the NMR spectra are not known but could account for the discrepancy of calculated and experimental results. The angular methyl resonance calculated for Structure III is very close to the values obtained for our unknown but the infrared spectrum of the unknown did not show any band at 1740 cm^{-1} to account for the 17-keto group. Further investigations are currently being carried out to elucidate the structure of this compound.

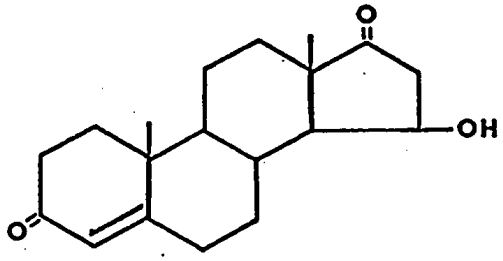
In order to confirm the isolation of 15α-hydroxyandrostenedione a second study was performed using a 6-day urine collection obtained from a normal subject in the 32nd week of gestation. In this study the isolation of 15α-hydroxytestosterone was also undertaken. To the 6-day urine pool were added



I $C_{18}H_{24}O_5$=320



II $C_{18}H_{24}O_5$=320



III $C_{19}H_{26}O_3$=302

FIG. 4. Possible structures of unknown steroid isolated from Pregnancy urine in Experiment A-2.

TABLE 23

Angular Methyl Resonance Calculations for Structures I, II and III, and Observed Values (ppm) for Unknown Isolated from Pregnancy Urine

	19-H				18-H			
	I	II	III	Unknown	I	II	III	Unknown
3-keto-Δ^4	1.210	1.210	1.210		0.770	0.770	0.770	
7β-OH		0.025				0.033		
12β-OH	0.008				0.067			
14-iso		-0.025				0.300		
14α-OH	0.0000				0.117			
14β-OH		0.017				-0.025		
15-keto		-0.042				0.192		
15β-OH			0.033				0.267	
17-keto	0.017		0.017		0.167		0.167	
	1.235	1.185	1.260	1.27	1.121	1.270	1.204	1.19
16-oxygen	?	?			?			

1.86×10^5 dpm of purified (4-^{14}C)-15α-hydroxyandrostenedione, specific activity 4.26×10^5 dpm/μg, and 1.50×10^5 dpm of (4-^{14}C)-15α-hydroxytestosterone, specific activity 4.23×10^5 dpm/μg. The urinary steroid conjugates were hydrolyzed with β-glucuronidase and the neutral extract obtained weighed 1.2 g and contained 2.50×10^5 dpm. It was chromatographed on a 120 g silica gel column using increasing concentrations of ethanol in methylene chloride. The effluent from the column was collected at a rate of 30-40 ml/hour and two peaks of radioactivity (Peaks A and B) were eluted with 3% and 8% ethanol in methylene chloride, respectively.

From Peak A an eluate was obtained which weighed 14 mg and contained 1.07×10^5 dpm. Chromatography on a 2 g alumina column and elution with 1.5% ethanol in benzene gave 9 mg of a residue containing 9.90×10^4 dpm. It was chromatographed on paper in system L and a single peak of radioactive material was obtained which after elution yielded a residue containing 9.40×10^4 dpm. Further purification was achieved by chromatography on paper in system J. The material eluted contained 7.40×10^4 dpm and it was chromatographed on a small alumina column. Elution with 1.5% ethanol in benzene gave 0.7 mg of residue which contained 6.50×10^4 dpm. This material was acetylated with ^{3}H-acetic anhydride, Batch No. 4, and the product was extracted with ethyl acetate after the addition of 60.0 mg carrier 15α-acetoxyandrostenedione. The mixture was reduced with $NaBH_4$ and the allylic alcohol at C-3 was then oxidized

with the DDQ reagent. Chromatography on a 6 g alumina column and elution with 0.5% ethanol in benzene gave 39.6 mg of material which was crystallized four times. Constant specific activities and $^{3}H/^{14}C$ ratios were obtained as shown in Table 24. The fourth crystals and mother liquor were combined

TABLE 24

Proof of Radiochemical Purity of 15α-Hydroxyandrostenedione Isolated from a 6-Day Collection of Pregnancy Urine

		Specific Activity (dpm ^{3}H/mg)		
Crystallization	Crystals	$^{3}H/^{14}C$	Mother Liquor	$^{3}H/^{14}C$
15α-Acetoxytestosterone*				
1	5440	7.5	28900	38.0
2	4300	6.0	6620	9.2
3	4200	5.8	5100	7.2
4	4090	5.8	4300	5.9
15α,17β-Diacetoxy-androst-4-en-3-one				
1	3680	5.8	3870	6.0
2	3680	5.8	3690	5.8
Calculated**	3730			

*After acetylation of the urinary 15α-hydroxyandrostenedione with ^{3}H-acetic anhydride, Batch No. 4, 60.0 mg of carrier 15α-acetoxyandrostenedione was added and the mixture was reduced with $NaBH_4$ and then oxidized with DDQ. Chromatography on alumina gave 39.6 mg of 15α-acetoxytestosterone which was crystallized.

**The fourth crystals and mother liquor were combined (15.0 mg) and acetylated. The diacetate was chromatographed on an alumina column to give an oil which weighed 16.0 mg and contained 5.97x104 dpm of ^{3}H. The calculated specific activity was based on these values.

(15.0 mg) and acetylated. The diacetate was chromatographed on a 2 g alumina column and elution with benzene-Skellysolve B (1:1) gave an oil (16.0 mg and 5.97×10^4 dpm of ^3H) which was crystallized to constant specific activity and constant $^3H/^{14}C$ ratio as shown in Table 24. From the final $^3H/^{14}C$ ratio and the specific activity of the acetic anhydride, the specific activity of the urinary 15α-hydroxyandrostenedione was found to be 6.93×10^3 dpm/μg. From this final specific activity it was calculated that the amount of 15α-hydroxyandrostenedione excreted in the urine was 4.4 μg/day.

The residue of Pool B (21 mg and 9.80×10^4 dpm), was chromatographed on a Whatman paper, No. 3MM, in system M, and one radioactive band was observed with the same mobility as 15α-hydroxytestosterone run alongside. An ultraviolet-absorbing band with the same mobility as the radioactive band was also observed. On elution, this radioactive material had 7.50×10^4 dpm and weighed 4.5 mg which was chromatographed on paper in system L. One radioactive band and one ultraviolet-absorbing band with identical mobilities were observed which on elution gave 6.05×10^4 dpm and 2.3 mg of residue. It was further purified by chromatography on paper in system J. Again, one radioactive band and one ultraviolet-absorbing band were observed which were eluted to yield a residue containing 4.80×10^4 dpm. This material was acetylated with ^3H-acetic anhydride, Batch No. 4, and after the addition of 37.5 mg of carrier 15α,17β-diacetoxyandrost-4-en-3-one the mixture was extracted with ethyl acetate. Chromatography on a 4 g alumina column and elution with benzene-

TABEE 25

Proof of Radiochemical Purity of 15α-Hydroxytestosterone Isolated from a 6-Day Collection of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
15α,17β-Diacetoxy-androst-4-en-3-one*				
1	49000	117	202500	470
2	43200	105	69200	176
3	42800	103	45300	108
15α,17β-Diacetoxy-androst-4-en-3β-ol				
1	39900	101	48500	118
2	40800	101	41200	103
Calculated**	42700			

*To the residue obtained after acetylation 37.5 mg of carrier 15α,17β-diacetoxy-androst-4-en-3-one was added prior to chromatography on alumina and crystallization of the material eluted.
**The third crystals (8.0 mg) were reduced with $NaBH_4$ and the product obtained was chromatographed on a thin layer plate in system Q. The 15α,17β-diacetoxy-androst-4-en-3β-ol eluted from the plate (7.2 mg, 2.99×10^5 dpm of ^3H, 2.76×10^3 dpm of ^{14}C) was then crystallized. The calculated specific activity was based on the weight and the ^3H content of the material eluted from the thin layer plate.

Skellysolve B (1:1) gave 31.5 mg of oily material which was crystallized to constant specific activity and $^3H/^{14}C$ ratio as shown in Table 25. The third crystals (8.0 mg) were reduced with $NaBH_4$ and the product obtained was chromatographed on a thin layer plate using system Q. On spraying a 0.5 cm portion with phosphomolybdic acid solution, one major spot was observed which was eluted to give a residue containing 2.99×10^5 dpm of ^3H and 2.76×10^3 dpm of ^{14}C and weighing 7.2 mg. This material

was crystallized from benzene-Skellysolve B-ether and from acetone-Skellysolve B and constant specific activities and $^{3}H/^{14}C$ ratios were obtained as shown in Table 25. The infrared spectrum (KBr) of the crystals showed retention of the acetate bands at 1250 and 1740 cm^{-1}, the absence of the α,β-unsaturated ketone band at 1670 cm^{-1} and the presence of the C=C band at 1610 cm^{-1}. Since it is known (39) that $NaBH_4$ reduction of a Δ^4-3-keto steroid gives predominantly a 3β-alcohol, this material most probably was 15α,17β-diacetoxy-androst-4-en-3β-ol. From the final $^{3}H/^{14}C$ ratio (Table 25) and the specific activity of the acetic anhydride, the specific activity of the urinary 15α-hydroxytestosterone was found to be 7.92×10^{2} dpm/μg. From this final specific activity it was calculated that the amount of 15α-hydroxytestosterone excreted in the urine was 31 μg/day.

SECTION B. ORIGIN OF 15α-HYDROXYPROGESTERONE

In Experiment A-1 15α-hydroxyprogesterone was isolated only from pregnancy urine and it could not be detected in the urine of four non-pregnant subjects examined. This finding suggested that 15α-hydroxyprogesterone was formed in the feto-placental unit. In the studies to be described in this section, progesterone labeled with tritium was injected into the peritoneal cavity of the fetus at the time of amniocentesis for Rh incompatibility and at the same time (4-^{14}C)-progesterone or (4-^{14}C)-15α-hydroxyprogesterone was injected in an antecubital vein of the mother. The maternal urine was then analyzed and 15α-hydroxyprogesterone was isolated and examined for its ^{3}H and ^{14}C contents.

These studies were done with the collaboration of Dr. J.M. Bowman, of the Rh Laboratory, Winnipeg, Manitoba. Urines were collected from the patients for 5 days, frozen and shipped to Montreal in this form.

The following three subjects were employed in these studies. All three subjects presented with Rh iso-immunization.

Subject GP: This woman had her first pregnancy in 1965 when she had a spontaneous delivery at 36 weeks of gestation. During her second pregnancy in 1967, she received two intrauterine blood transfusions. The first transfusion at 29 weeks 5 days of gestation consisted of 96 ml (23.7 g of hemoglobin) of type O Rh negative packed red blood cells. At 31 weeks 1 day of gestation she received a second intrauterine transfusion using

100 ml (29.7 g of hemoglobin) of type O Rh negative packed red blood cells mixed with ^{3}H-progesterone. During this transfusion ^{14}C-progesterone was injected intravenously to the mother and urine was collected for 5 days after the injection and used in these studies. Following Pitocin induction and vaginal delivery at 34 weeks 6 days of gestation she had a 4 lb. 4 oz. baby. The cord hemoglobin was 12.5 g% and the cord bilirubin was 6.1 mg%. The cord blood was Coombs negative. Only one fetal cell was found in 5,000 adult donor cells by the Kleihauer technique. A sudden episode of anoxia appeared at 10 minutes of age. After initial exchange transfusion the baby developed a progressive respiratory distress and was placed on Bennet respiration and intravenous administration of dextrose and bicarbonate. The results of an X-ray examination were consistent with hyaline membrane disease. The baby died at 48 hours of age. Post mortem examination showed classical hyaline membrane disease and minimal, if any, hepatic dysfunction.

Subject AB: The first pregnancy of this subject, in 1962, was normal. Rh iso-immunization complications were first found in 1963, during her second pregnancy. Induction and vaginal delivery was carried out at 37 weeks of gestation. The baby, a male, weighed 5 lb. 8 1/2 oz. and required two exchange transfusions. During her third pregnancy, in 1967, she received two intrauterine blood transfusions. The initial transfusion was carried out at 28 weeks 6 days of gestation. At 30 weeks 5 days

of gestation she received a second intrauterine transfusion using 110 ml (28.8 g of hemoglobin) of type O Rh negative packed red blood cells mixed with ^{3}H-progesterone. During this transfusion ^{14}C-progesterone was injected intravenously to the mother and urine was collected for 5 days and used in these studies. Induction and vaginal delivery was carried out at 34 weeks 5 days of gestation. The baby, a male, weighed 5 lb. 4 oz. and had 11.4 g% cord hemoglobin and 5.7 mg% of cord bilirubin. He required six exchange transfusions. The cord blood was weakly Coombs positive. About 76% of his cord blood cells were adult donor cells as estimated by the Kleihauer cell staining technique. He is alive and well.

Subject SP: The first two pregnancies of this woman (1954 and 1956) were normal. Rh iso-immunization complications were first found in 1958, during her third pregnancy. Induction and vaginal delivery was carried out at 37 weeks of gestation. The baby required 5 exchange transfusions and is alive and well. In 1962, she had a fetal loss at 17 weeks of gestation. During her fifth pregnancy, in 1968, she received three intrauterine blood transfusions at 25 weeks, 26 weeks 4 days, and 29 weeks 3 days of gestation, respectively. At the time of the second transfusion the fetus was hydropic, intraperitoneal digoxin was instilled and the mother was placed on digoxin. The third intrauterine transfusion consisted of 100 ml (25.4 g of hemoglobin) of type O Rh negative packed red blood cells mixed with ^{3}H-progesterone. Simultaneously with the transfusion

(4-^{14}C)-15α-hydroxyprogesterone was injected intravenously to the mother and urine was collected for 5 days and used in these studies. Following induction with Pitocin at 33 weeks 5 days of gestation, she failed to go into labour and delivery was carried out 5 days later by Caesarian section. The baby, a male, weighed 5 lb. 6 oz. and had 9.9 g% cord hemoglobin and 7.0 mg% cord bilirubin. He required two exchange transfusions and his cord blood was weakly Coombs positive. About 94% of the cord blood cells were adult donor cells as estimated by the Kleihauer technique. At two weeks of age he was convalescent and clinically well, but his total bilirubin was 11 mg% and there was evidence of hepato-cellular damage.

In each case tritiated progesterone was introduced into the peritoneal cavity of the fetus during a transfusion, in utero, for erythroblastosis fetalis. The method for the injection of ^{3}H-progesterone during blood transfusion was described in a previous section of this thesis. Simultaneously with the injection of tritiated progesterone, ^{14}C-progesterone was administered in an antecubital vein of subjects GP and AB and ^{14}C-15α-hydroxyprogesterone was injected in subject SP.

The doses administered are shown in Table 26. Urine was collected for 5 days and each day's urine was solvolyzed and then hydrolyzed with β-glucuronidase. The total radioactivity recovered in each day's urine and the amount present as sulfates and glucosiduronates are shown in Tables 27-29.

The glucosiduronate fraction obtained from the urine of subject GP weighed 1.27 g and contained 9.65×10^{5} dpm of ^{3}H

TABLE 26

Amount of Radioactivity and Nature of the Steroid Injected into the Peritoneal Cavity of the Fetus and the Maternal Vein

Subject	Steroid Injected into the Peritoneal Cavity of the Fetus	Dose Injected (dpm)	Steroid Injected into the Maternal Vein	Dose Injected (dpm)
GP	^{3}H-Progesterone	6.05×10^{6}	^{14}C-Progesterone	3.85×10^{6}
AB	^{3}H-Progesterone	4.73×10^{6}	^{14}C-Progesterone	2.62×10^{6}
SP	^{3}H-Progesterone	4.97×10^{6}	^{14}C-15α-Hydroxy-progesterone	9.00×10^{3}

and 1.66×10^{6} dpm of ^{14}C. It was chromatographed on a 120 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described and the radioactivity in the eluted fractions is shown in Figure 5A. The weight of the radioactive material and the radioactivity of each pool are shown in Table 30.

TABLE 27

Recovery of Radioactivity in the Urine and the Extracts Obtained After Hydrolysis of Conjugates by Solvolysis and by β-Glucuronidase. Subject GP

	Crude Urine (dpm)		% Recovery of Injected Dose	
Day	^{3}H	^{14}C	^{3}H	^{14}C
1	6.90×10^{5}	1.42×10^{6}	11.4	36.9
2	1.90×10^{5}	2.45×10^{5}	3.1	6.4
3	1.60×10^{5}	1.15×10^{5}	2.7	3.0
4	8.20×10^{4}	5.30×10^{4}	1.4	1.4
5	2.30×10^{4}	2.70×10^{4}	0.4	0.7
Total	1.15×10^{6}	1.86×10^{6}	19.0	48.4
	Glucosiduronates (dpm)		% Recovery of Injected Dose	
	^{3}H	^{14}C	^{3}H	^{14}C
1	5.83×10^{5}	1.30×10^{6}	9.6	33.8
2	1.48×10^{5}	1.94×10^{5}	2.5	5.0
3	1.40×10^{5}	9.90×10^{4}	2.3	2.6
4	7.50×10^{4}	4.30×10^{4}	1.2	1.1
5	1.90×10^{4}	2.00×10^{4}	0.3	0.5
Total	9.65×10^{5}	1.66×10^{6}	15.9	43.0
	Sulfates (dpm)		% Recovery of Injected Dose	
	^{3}H	^{14}C	^{3}H	^{14}C
1	4.59×10^{4}	5.31×10^{4}	0.8	1.4
2	2.33×10^{4}	3.28×10^{4}	0.4	0.9
3	5.00×10^{3}	9.00×10^{3}	0.1	0.2
4	-	3.00×10^{3}	-	0.1
5	-	-	-	-
Total	7.40×10^{4}	9.79×10^{4}	1.3	2.6

TABLE 28

Recovery of Radioactivity in the Urine and the Extracts Obtained After Hydrolysis of Conjugates by Solvolysis and by β-Glucuronidase. Subject AB

Day	Crude Urine (dpm) ^3H	Crude Urine (dpm) ^{14}C	% Recovery of Injected Dose ^3H	% Recovery of Injected Dose ^{14}C
1	7.01×10^5	6.42×10^5	14.8	24.5
2	1.21×10^5	7.05×10^4	2.6	2.7
3	2.80×10^4	1.85×10^4	0.6	0.7
4	1.32×10^4	7.60×10^3	0.3	0.3
5	5.00×10^3	3.20×10^3	0.1	0.1
Total	8.68×10^5	7.42×10^5	18.4	28.3
	Glucosiduronates (dpm) ^3H	Glucosiduronates (dpm) ^{14}C	% Recovery of Injected Dose ^3H	% Recovery of Injected Dose ^{14}C
1	5.52×10^5	4.82×10^5	11.7	18.4
2	8.81×10^4	4.64×10^4	1.9	1.8
3	1.84×10^4	1.02×10^4	0.4	0.4
4	8.00×10^3	4.00×10^3	0.2	0.2
5	-	-	-	-
Total	6.61×10^5	5.43×10^5	14.2	20.8
	Sulfates (dpm) ^3H	Sulfates (dpm) ^{14}C	% Recovery of Injected Dose ^3H	% Recovery of Injected Dose ^{14}C
1	8.93×10^4	1.08×10^5	1.9	4.1
2	1.97×10^4	1.62×10^4	0.4	0.6
3	4.50×10^3	4.10×10^3	0.1	0.2
4	2.60×10^3	2.53×10^3	0.1	0.1
5	-	-	-	-
Total	1.16×10^5	1.31×10^5	2.5	5.0

TABLE 29

Recovery of Radioactivity* in the Urine and the Extracts Obtained After Hydrolysis of Conjugates by Solvolysis and by β-Glucuronidase. Subject SP

Day	Crude Urine (dpm of ^3H)	% Recovery of Injected Dose
1	8.75×10^5	17.6
2	1.94×10^5	3.9
3	1.01×10^5	2.0
4	6.20×10^4	1.2
5	2.60×10^4	0.5
Total	1.26×10^6	25.2
	Glucosiduronates (dpm of ^3H)	**% Recovery of Injected Dose**
1	7.05×10^5	14.2
2	1.48×10^5	3.0
3	6.30×10^4	1.3
4	3.30×10^4	0.7
5	1.70×10^4	0.3
Total	9.66×10^5	19.5
	Sulfates (dpm of ^3H)	**% Recovery of Injected Dose**
1	6.80×10^4	1.4
2	2.30×10^4	0.6
3	1.80×10^4	0.4
4	-	-
5	-	-
Total	1.09×10^5	2.4

*Since only 9,000 dpm of (4-^{14}C)-15α-hydroxyprogesterone and 4.97×10^6 dpm of ^3H-progesterone (injected $^3H/^{14}C$ ratio = 552) were injected, ^{14}C could not be detected in crude urine and the neutral extracts.

TABLE 30

Weight and Radioactivity in Fractions Eluted from the Initial Silica Gel Column Obtained after Chromatography of the Extract from the Glucosiduronate Fraction. Subject GP

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	^{14}C
I	1-62	18	-	-
II	63-102	175	9.92×10^{4}	2.47×10^{5}
III	103-134	229	6.00×10^{4}	1.14×10^{5}
IV	135-162	134	1.71×10^{5}	4.63×10^{5}
V	163-220	213	1.54×10^{5}	3.68×10^{5}
VI	221-302	116	2.80×10^{4}	2.04×10^{4}
VII	303-350	58	5.05×10^{4}	5.24×10^{4}
VIII	351-448	159	1.55×10^{5}	1.40×10^{5}
IX	449-626	163	1.10×10^{5}	5.76×10^{4}

Pool VI, Figure 5A, Table 30, gave a residue which was chromatographed on a 10 g alumina column. Elution with 1.5% ethanol in benzene gave 56.0 mg of material which contained 2.32×10^{4} dpm of ^{3}H and 1.34×10^{4} dpm of ^{14}C. It was further chromatographed on a Whatman No. 3MM paper in system A for 10 hours and three radioactive zones, VIA, VIB and VIC were obtained with mobilities of 5.0, 15.5 and 19.3 cm, respectively. The radioactive material in zone VIB had a mobility identical to an ultraviolet-absorbing band observed on the paper and with 15α- and 16α-hydroxyprogesterone standards run alongside. In

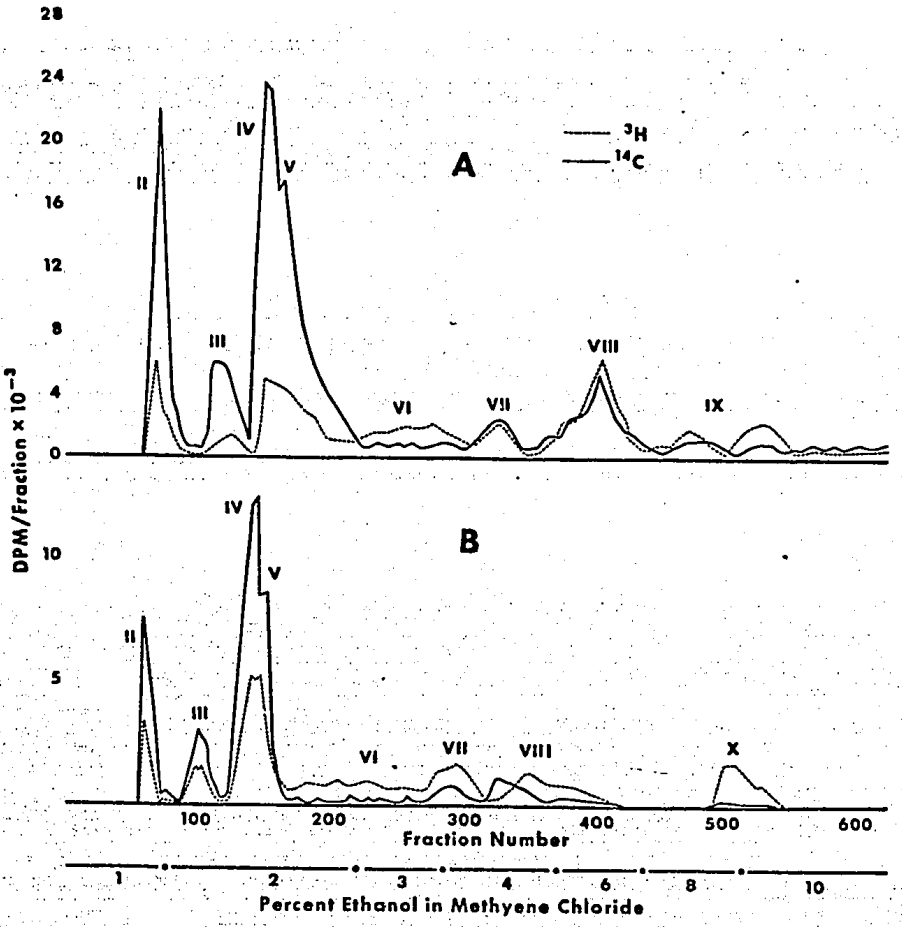


FIG. 5. Silica gel column chromatography of the glucosiduronate fractions of the urine of subjects GP and AB following the simultaneous administration of ^{3}H-progesterone to the peritoneal cavity of the fetus and ^{14}C-progesterone to the mother.

A. Subject GP
B. Subject AB

this system 15α- and 16α-hydroxyprogesterone are not separated. Zone VIB was eluted (5.03×10^3 dpm of ^3H and 1.75×10^3 dpm of ^{14}C) and the material contained therein was chromatographed on paper in system B for 5 days. Two ultraviolet-absorbing zones, VIBI (10 cm) and VIBII (12 cm) were obtained having mobilities identical to 15α- and 16α-hydroxyprogesterone, respectively. On elution, zone VIBI gave a residue containing 1,200 dpm of ^3H and 500 dpm of ^{14}C.

The glucosiduronate fraction obtained from the urine of subject AB weighed 1.35 g and contained 6.66×10^5 dpm of ^3H and 5.43×10^5 dpm of ^{14}C. It was chromatographed on a 120 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. In Figure 5B is shown the eluted radioactive material from which the different pools shown in Table 31 were obtained.

The residue contained in Pool VI, Figure 5B, Table 31, was chromatographed on an 8 g alumina column and elution with 1.5% ethanol in benzene gave a residue which weighed 52.0 mg and contained 2.50×10^4 dpm of ^3H and 8.30×10^3 dpm of ^{14}C. It was further chromatographed on a Whatman No. 3MM paper in system A for 10 hours and three radioactive zones, VIA, VIB and VIC were obtained with mobilities of 6.0, 19.0 and 22.3 cm, respectively. The radioactive material in zone VIB had a mobility identical to an ultraviolet-absorbing band observed on the paper and to 15α- and 16α-hydroxyprogesterone standards run alongside. The material present in zone VIB was eluted (7.20×10^3 dpm of ^3H and 2.80×10^3 dpm of ^{14}C) and it was chromatographed on paper in system B for 5 days. Two ultraviolet-absorb-

TABLE 31

Weight and Radioactivity in Fractions Eluted from the Initial Silica Gel Column after Chromatography of the Extract from the Glucosiduronate Fraction. Subject AB

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	^{14}C
I	1-56	28	-	-
II	57-90	153	7.11×10^{4}	7.89×10^{4}
III	91-116	222	2.45×10^{4}	3.21×10^{4}
IV	117-146	94	1.11×10^{5}	1.71×10^{5}
V	147-166	55	5.34×10^{4}	7.86×10^{4}
VI	167-274	126	2.74×10^{4}	1.09×10^{4}
VII	275-310	45	4.01×10^{4}	2.56×10^{4}
VIII	311-406	147	1.21×10^{5}	5.84×10^{4}
IX	407-490	54	3.68×10^{4}	1.38×10^{4}
X	491-540	39	9.01×10^{4}	1.08×10^{4}

ing bands, VIBI and VIBII, were observed with mobilities corresponding to 15α- and 16α-hydroxyprogesterone, respectively. After elution, the material from zone VIBI gave a residue containing 1700 dpm of ^{3}H and 800 dpm of ^{14}C.

Because the material having the chromatographic mobility of 15α-hydroxyprogesterone had insufficient amounts of radioactivity for a test of purity by the isotope dilution procedure, the two residues (residue VIBI isolated from pool

VI, Figure 5A, Table 30, and residue VIBI, Pool VI, Figure 5B, Table 31) isolated from the urine of subjects GP and AB, respectively, were combined and processed together. The combined fraction weighed 3.1 mg and contained 2900 dpm of ^{3}H and 1300 dpm of ^{14}C. After the addition of 6.1 mg of carrier 15α-hydroxyprogesterone, the mixture was chromatographed on a 1 g alumina column and 6.5 mg of crystalline material was eluted with 1.5% ethanol in benzene. This material was crystallized to constant specific activity as shown in Table 32. The steroid used for counting the first, second and third crystals and the third mother liquor was recovered from the counting vials and combined with the remaining material from the third crystallization. The mixture (4.0 mg) was acetylated with non-labeled acetic anhydride and the product was chromatographed on a 1 g alumina column. Elution with benzene-Skellysolve B (4:1) gave 3.5 mg of oily material which was crystallized to constant specific activity as shown in Table 32.

The glucosiduronate fraction obtained from the urine of subject SP weighed 1.82 g and contained 9.66×10^{5} dpm of ^{3}H. No ^{14}C could be detected in the extracts at this stage due to the high $^{3}H/^{14}C$ ratio (552) injected. To this fraction was added 3.0 mg of carrier 15α-hydroxyprogesterone and the mixture was chromatographed on a 160 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. In Figure 6 is shown the eluted radio-

TABLE 32

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Combined Residues Obtained from the Urine of Subjects GP and AB

	Specific Activity (dpm/mg)			
	Crystals		Mother Liquor	
Crystallization	^{3}H	^{14}C	^{3}H	^{14}C
15α-Hydroxyprogesterone				
1	330	100	450	430
2	260	60	600	220
3	250	50	260	60
Calculated*	480	210		
15α-Acetoxyprogesterone				
1	220	20	240	150
2	220	10	230	20
Calculated**	220	40		

*The residues obtained from Pool VI, Figure 5A, Table 30, and from Pool VI, Figure 5B, Table 31, were combined (3.1 mg, 2900 dpm of ^{3}H, 1300 dpm of ^{14}C) and processed together. To the combined fraction was added 6.1 mg of carrier 15α-hydroxyprogesterone and the mixture was chromatographed on a 1 g alumina column prior to crystallization.

**The third crystals and the material recovered from the counting vials of the first, second and third crystals and the third mother liquor, were combined and the mixture was acetylated. The calculated specific activity of the acetate was based on the final specific activity of 15α-hydroxyprogesterone and altered molecular weight.

active material from which the different pools shown in Table 33 were obtained.

The residue contained in Pool IV, Figure 6, Table 33, was chromatographed on a 12 g alumina column and elution with

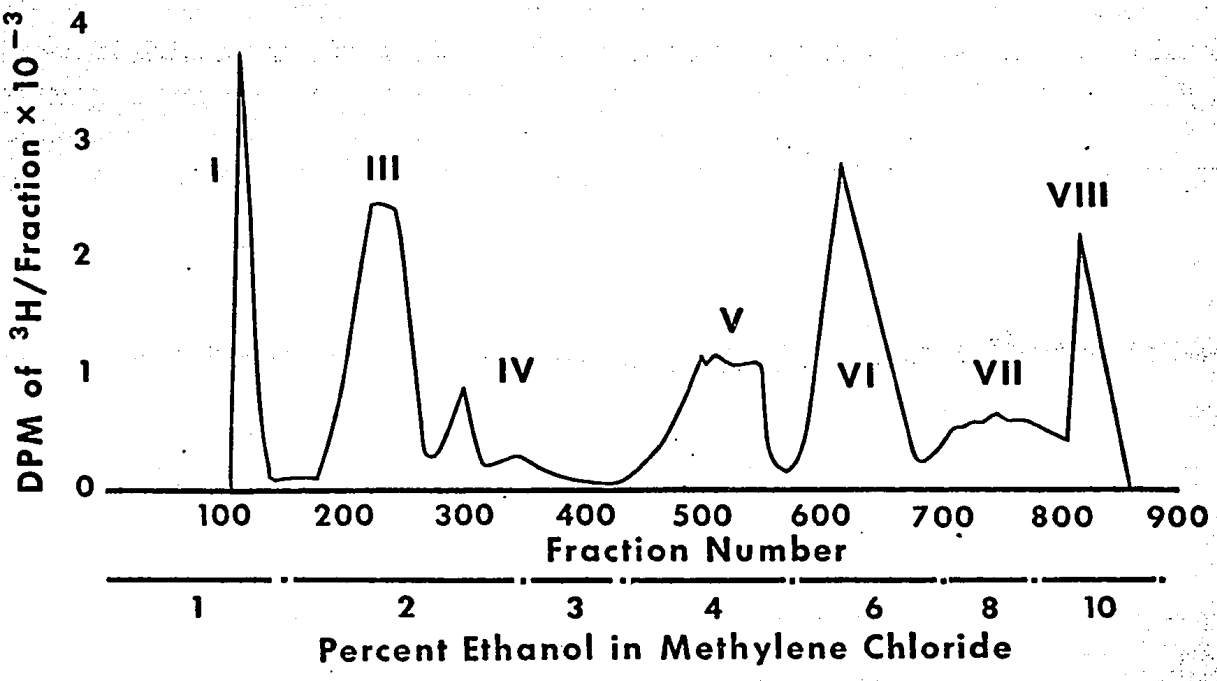


FIG. 6. Silica gel column chromatography of the Glucosiduronate fraction of theurine of Subject SP following the simultaneous administration of ^{3}H-progesterone to the peritoneal cavity of the fetus and (4-^{14}C)-15α-hydroxyprogesterone to the mother. Since only 9000 dpm of (4-^{14}C)-15α-hydroxyprogesterone was injected, ^{14}C could not be detected at this stage.

TABLE 33

Weight and Radioactivity in Fractions Eluted from the Initial Silica Gel Column after Chromatography of the Extract from the Glucosiduronate Fraction of the Urine. Subject SP

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	^{14}C
I	111-141	164	6.40×10^{4}	-
II	142-184	60	1.60×10^{4}	-
III	185-268	385	1.80×10^{5}	-
IV	269-432	125	3.40×10^{4}	2.90×10^{3}
V	433-568	175	1.04×10^{5}	1.40×10^{3}
VI	569-679	203	1.60×10^{5}	9.00×10^{2}
VII	680-806	667	8.80×10^{4}	-
VIII	807-859	41	8.50×10^{4}	4.00×10^{2}

1.5% ethanol in benzene gave a residue which weighed 11.5 mg and contained 1.01×10^{4} dpm of ^{3}H and 2.77×10^{3} dpm of ^{14}C. It was further chromatographed on a Whatman No. 3MM paper in system A for 12 hours and two radioactive zones, IVA and IVB were obtained with mobilities of 12.0 and 32.0 cm, respectively. The radioactive material in zone IVB had a mobility identical to an ultraviolet-absorbing band observed on the paper and to 15α- and 16α-hydroxyprogesterone standards run alongside. The material present in zone IVB was eluted (6.60×10^{3} dpm of ^{3}H and 2.30×10^{3} dpm of ^{14}C) and it was chromatographed on paper

in system P for 4 days. Two ultraviolet-absorbing bands, IVBI and IVBII, were observed with mobilities corresponding to 15α- and 16α-hydroxyprogesterone, respectively. After elution, the material from zone IVBI gave a residue which weighed 3.8 mg and contained 1400 dpm of ^{3}H and 2200 dpm of ^{14}C. It was further purified by chromatography on a 1 g alumina column and elution with 1.5% ethanol gave 2.5 mg of residue containing 1360 dpm of ^{3}H and 2100 dpm of ^{14}C. The infrared spectrum (KBr) of an aliquot (60 μg) of this material was identical to that of 15α-hydroxyprogesterone. Since only small amounts of 15α-hydroxyprogesterone (30 μg/day) are present in human late pregnancy urine, it can be assumed that the 2.5 mg of 15α-hydroxyprogesterone present in zone IVBI represents a fraction of the 3.0 mg of 15α-hydroxyprogesterone which was added to the glucosiduronate fraction of the urine prior to the initial silica gel column chromatography. The material isolated from fraction IV was mixed with an additional 4.0 mg of carrier 15α-hydroxyprogesterone and the mixture (6.5 mg) was crystallized to constant specific activities and constant $^{3}H/^{14}C$ ratio as shown in Table 34. The steroid used for counting the first and second crystals and the second mother liquor was recovered from the counting vials and combined with the remaining material from the second crystallization. The mixture (4.5 mg) was acetylated with non-labeled acetic anhydride and the product was chromatographed on a 1 g alumina column. Elution with benzene-Skellysolve B (4:1) gave 4.2 mg of oily

TABLE 34

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Glucosiduronate Fraction of the Urine of Subject SP

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyprogesterone						
1	160	300	0.53	400	280	1.43
2	150	310	0.48	170	320	0.53
Calculated*	210	320				
15α-Acetoxyprogesterone						
1	110	270	0.41	180	280	0.64
2	110	260	0.42	120	270	0.44
Calculated**	130	270				

*The material isolated from fraction IV, Figure 6, Table 33, weighed 2.5 mg and contained 1360 dpm of ^{3}H and 2100 dpm of ^{14}C. It was mixed with an additional 4.0 mg of carrier 15α-hydroxyprogesterone (thus a total of 6.5 mg of carrier steroid was added to fraction IV, see text for details) and crystallized.

**The steroid used for counting the first and second crystals and the second mother liquor was recovered from the counting vials and combined with the remaining material from the second crystallization. The mixture was then acetylated and the product chromatographed on an alumina column prior to crystallization. The calculated specific activity of the acetate was based on the final specific activity of 15α-hydroxyprogesterone and altered molecular weight.

material which was crystallized to constant specific activity and constant $^{3}H/^{14}C$ ratio as shown in Table 34.

SECTION C. METABOLISM OF 15α-HYDROXYPROGESTERONE

Experiment C-1. Metabolism of 15α-Hydroxyprogesterone by the Normal Male

Steroids having a 15α-hydroxyl group are not readily available and are difficult to synthesize. As a result this study was designed to obtain large amounts of urinary metabolites of 15α-hydroxyprogesterone. The subject, a 28-year old male, was given 2.30×10^7 dpm of (7-^{3}H)-15α-hydroxyprogesterone in 1.32 mg by intravenous injection. At the same time 800 mg of 15α-hydroxyprogesterone was administered orally, in 80 mg doses contained in gelatin capsules, over a period of ten hours. After the injection of the labeled steroid, urine was collected for six days and the steroid conjugates contained in the individual urines were hydrolyzed with Glusulase. The total radioactivity recovered in each day's urine and the amount present in the neutral extracts after hydrolysis are shown in Table 35. Since almost all the radioactivity was present in the first four days the extracts from this period were combined and processed further.

The combined 4-day urine extract weighed 1.12 g and contained 1.62×10^7 dpm. It was chromatographed on a 150 g silica gel column which was developed with increasing concentrations of ethanol in methylene chloride as previously described and the radioactivity in the eluted fractions is shown in Figure 7.

TABLE 35

Recovery of Radioactivity in the Urine and the Extracts Obtained After Hydrolysis of Conjugates by Glusulase

Day	Crude Urine (dpm)	% Recovery of Injected Dose	Extracts After Hydrolysis	% Recovery of Injected Dose
1	1.51×10^7	65.7	1.36×10^7	59.2
2	2.84×10^6	12.3	2.15×10^6	9.3
3	4.05×10^5	1.8	2.78×10^5	1.2
4	1.56×10^5	0.7	1.09×10^5	0.5
5	-	-	3.57×10^4	0.2
6	-	-	1.23×10^4	0.1
Total	1.85×10^7	80.5	1.62×10^7	70.5

The combinations of the fractions eluted and the radioactivity and weight of the residue of each pool are shown in Table 36. The residue of pool IV was chromatographed on a 100 g Celite column using system E and two peaks of radioactive material were eluted from the column as shown in Figure 8a. Chromatography of the residue from IVA on paper in system A for 12 hours provided one radioactive zone with an average mobility of 7.9 cm. On elution a residue was obtained (18 mg and 3.20×10^5 dpm) which was further purified by chromatography on a 2 g silica gel column to give 3.3 mg of material containing 2.99×10^5 dpm. Crystallization of this material from acetone-methanol gave 0.5 mg of coarse needles. The infrared spectrum (KBr) of the

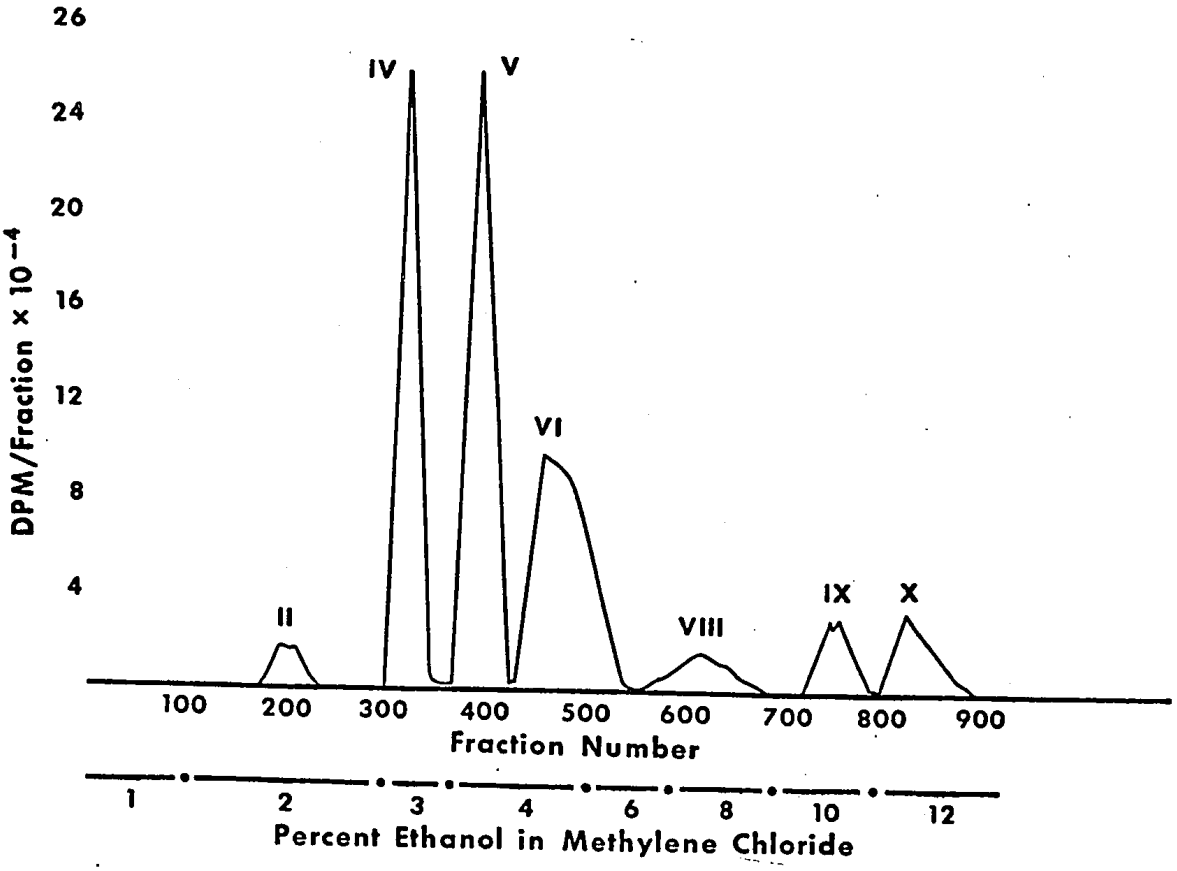


FIG. 7. Silica gel chromatography of urinary neutral extract following the intravenous injection of (7-^{3}H)-15α-hydroxyprogesterone and the oral administration of carrier steroid to a normal male.

TABLE 36

Weight and Radioactivity eluted from the Initial Silica Gel Column after Chromatography of the Extract Obtained by Hydrolysis of the Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	1-188	145	
II	189-212	157	1.54×10^5
III	213-300	56	2.05×10^5
IV	301-351	80	3.94×10^6
V	352-420	81	4.52×10^6
VI	421-540	117	3.94×10^6
VII	541-560	20	8.20×10^4
VIII	561-700	53	5.82×10^5
IX	701-800	29	6.60×10^5
X	801-890	29	1.42×10^6

crystals showed a strong hydroxyl band at 3456 cm^{-1} and a band at 1695 cm^{-1} corresponding to a 20-ketone. This material was not identified because the amount isolated was insufficient for mass spectrum and NMR analysis.

An aliquot of residue IVB, Figure 8a, (2.7 mg, 1.93×10^5 dpm) was chromatographed on paper in system A and one radioactive peak was obtained with an average mobility of 18.0 cm. Therefore this material was eluted from the paper and combined with the remainder of residue IVB, Figure 8a. Following chromato-

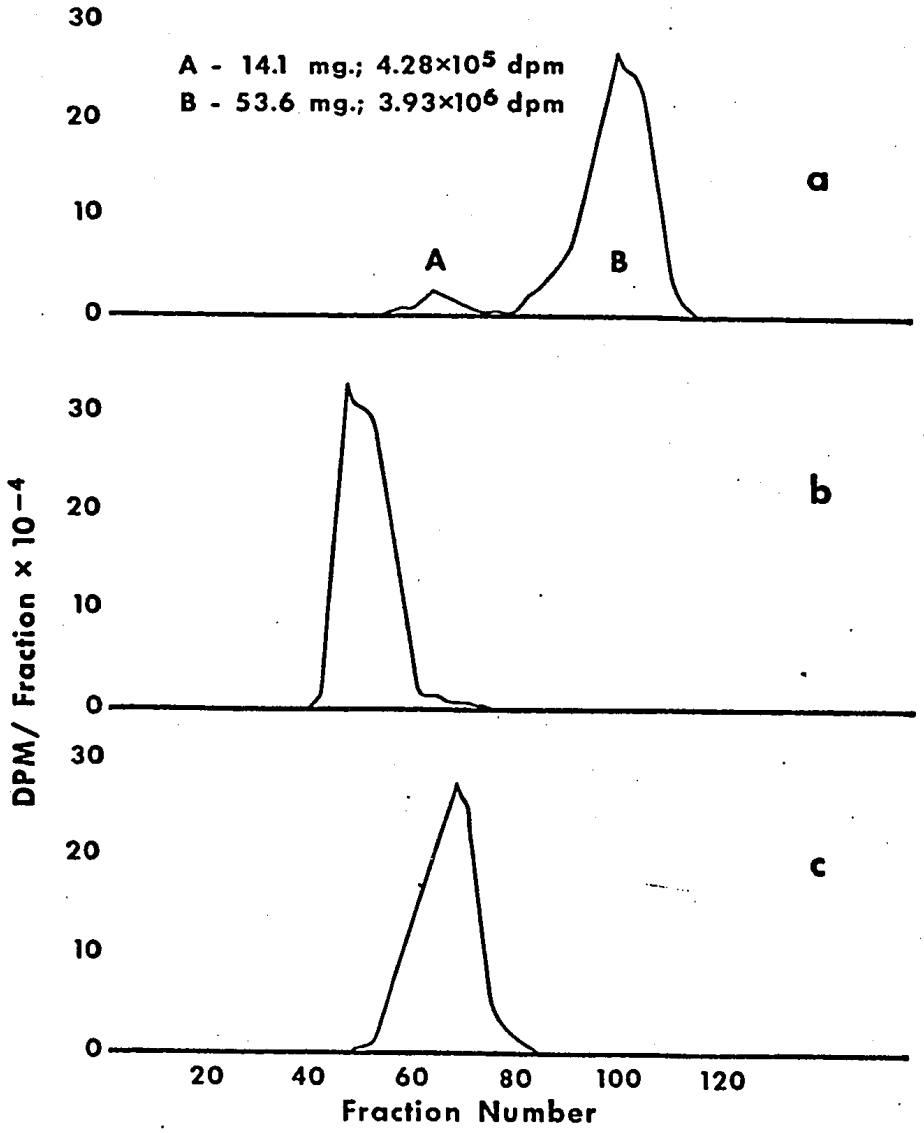


FIG. 8. Celite partition column chromatography of residues in Pools IV, V and VI in Experiment C-1.

a. Residue of Pool IV, System E
b. Residue of Pool V, System F
c. Residue of Pool VI, System F

graphy on a 6 g alumina column, 43.3 mg of crystalline material was obtained containing 3.62×10^6 dpm. Crystallization of this material from acetone gave 27.4 mg of coarse needles. The crystals had a mp of 230-232°C, and the specific activity was 1.08×10^5 dpm/mg. The infrared spectrum (KBr) of the crystals was identical to that of authentic 15α-hydroxyprogesterone.

Pool V, Table 36, Figure 7, yielded a residue which was chromatographed on a 100 g Celite column using system F. One main peak of radioactivity was obtained as shown in Figure 8b. The material within this peak was pooled to give 56 mg of residue which contained 4.48×10^6 dpm. An aliquot of this material (2.8 mg) was chromatographed on paper in system A for 24 hours and one radioactive peak was obtained with an average mobility of 22.9 cm. It was therefore eluted and combined with the remainder of the residue of V. Chromatography of this material on a 6 g silica gel column yielded 51.4 mg of an oily residue which contained 4.45×10^6 dpm. It was crystallized with great difficulty from Skellysolve B-ether-acetone to yield 14.0 mg of fine plates: mp 175-177°C, specific activity 1.42×10^5 dpm/mg. An aliquot of V containing 8.3 mg was acetylated with ^{14}C-acetic anhydride, solution No. 2, and the product was chromatographed on a small alumina column. Elution with 50-60% benzene in Skellysolve B gave 10.2 mg of colorless oil which was crystallized from acetone-Skellysolve B to yield 6.5 mg of coarse plates, mp 142-144°C. The specific

activity of the crystals was 4.08×10^4 dpm ^{14}C/mg ($^3H/^{14}C$ = 1.98), indicating the presence of two acylable hydroxyl groups. The infrared spectra of the free compound (KBr) and of the acetate (CS_2) are shown in Figure 9. The infrared spectrum of the free compound showed a hydroxyl band at 3350 cm^{-1} and a carbonyl band at 1705 cm^{-1} corresponding to a 20-ketone. The spectrum of the acetate had no hydroxyl band indicating that all hydroxyl groups on the molecule had been acetylated. At this point 2.7 mg of the unacetylated compound V and 4.8 mg of the acetate were sent to Dr. L. Durham at Stanford (Dr. Djerassi's laboratory), Stanford, California, for mass spectrum and nuclear magnetic resonance analyses. The results obtained from the NMR analysis are shown in Table 37. The C-21-$COCH_3$ signal was observed at 2.10 ppm. The acetate had two additional methyl signals at 1.98 and 2.03 ppm. The chemical shifts of the C-18 and C-19 methyl-hydrogens (Table 37) of the unacetylated compound and its acetate more closely resemble those of 5α-steroids. Using this as a starting point, and assuming that the acyl group at C-17 remains β, it is possible to calculate positions for C-18 and C-19 absorptions for different likely structures from the additive substituent effects given in Zurcher's Tables (45). The calculations for the 3α,15α-diol and the diacetate in the 5α,14α-series agree quite well (Table 37). The results for either 3α,15β-diol or 3β, 15β-diol were sufficiently far off to render it unlikely that anything had happened at C-15. However, the results for 3β,15α-diol

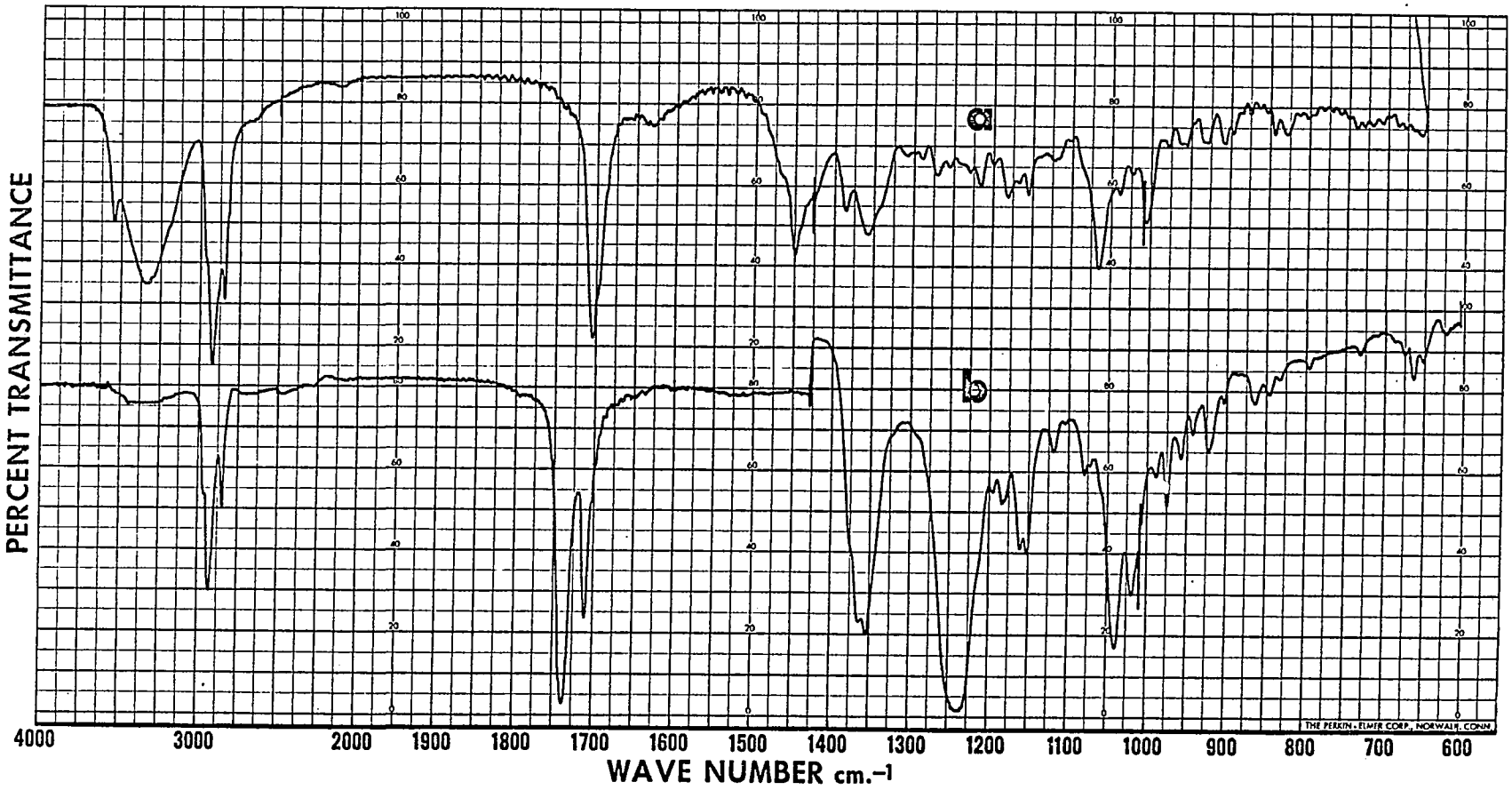


FIG. 9. (a) Infrared spectrum (KBr) of metabolite isolated from Pool V of neutral extract of urine.
(b) Infrared spectrum (CS_2) of the acetate of the metabolite isolated from Pool V.

TABLE 37

Observed Chemical Shifts, δ (PPM), of Metabolite V and its Diacetate and Calculated C-19 and C-18 Chemical Shifts of 3α,15α-Dihydroxy-5α-pregnan-20-one and its Diacetate

	Observed Chemical Shifts					
Compound	C-19	C-18	C-21-$COCH_3$	CH_3COO	C-3-H	Splitting Pattern of C-3-H
V	0.79	0.63	2.10	-	3.9-4.2	equatorial
V-diacetate	0.79	0.68	2.10	1.98,2.03	4.8-5.1	equatorial

Compound	Calculated Chemical Shifts	
	C-19	C-18
3α,15α-Dihydroxy-5α-pregnan-20-one	0.792	0.650
3α,15α-Diacetoxy-5α-pregnan-20-one	0.809	0.693

were quite close to those obtained for V, but they did not fit as well as the results for the 3α,15α-diol. If the assignment 3α,15α-diol is correct, then the C_3-H should be equatorial and hence the C_3-H adsorption should be sharper due to both axial-equatorial and equatorial-equatorial coupling being of medium size, and to lower field. The NMR spectrum of the free compound had the two low-field protons superimposed at 3.9-4.2 ppm and that of the acetate at 4.8-5.1 ppm; but the pattern had the appearance of a sharper signal atop a broader one.

From the NMR results (Table 37) combined with the information obtained from the infrared spectra, the number of acylable hydroxyl groups and from the mass spectrum (courtesy of Dr. L. Durham) of the free compound (m/e = 334), the structure 3α,15α-dihydroxy-5α-pregnan-20-one can be assigned to the metabolite isolated from pool V.

The residue from pool VI, Table 36, Figure 7, was chromatographed on a 100 g Celite column using system F. One major peak of radioactivity was obtained (Figure 8c) which contained 60.3 mg of crystalline material and had 3.62×10^6 dpm. An aliquot of this material (3.0 mg) was chromatographed on paper in system A for 24 hours and one band of radioactive material with an average mobility of 20.5 cm was obtained. It was eluted and rechromatographed on paper in system G for 55 hours and again one radioactive peak at an average distance of 16.5 cm was observed. Therefore this material was eluted

and combined with the remainder of residue VI. Chromatography on a 6 g silica gel column and elution with 4% ethanol in methylene chloride afforded 43.0 mg of crystalline material containing 3.33×10^6 dpm. It was crystallized from acetone-methanol-Skellysolve B to yield 23.0 mg of coarse plates: mp 208-210°C, specific activity 7.90×10^4 dpm/mg. Its infrared spectrum (KBr) showed a strong hydroxyl band at 3375 cm^{-1} and a band at 1705 cm^{-1} corresponding to a 20-ketone (Figure 10). An aliquot of VI containing 9.3 mg was acetylated with ^{14}C-acetic anhydride, Solution No. 2, and the residue from the reaction mixture was chromatographed on a small alumina column. Elution with 50-60% benzene in Skellysolve B gave 12.0 mg of colorless oil which was crystallized from acetone-Skellysolve B to yield 10.9 mg of fine needles: mp 164-166.5°C, specific activity 3.89×10^4 dpm ^{14}C/mg ($^3H/^{14}C$ = 1.65) indicating the presence of two acylable hydroxyl groups. The infrared spectrum (CS_2) of the acetate had no hydroxyl band (Figure 10) indicating that all hydroxyl groups on the molecule had been acetylated. The results of the NMR analysis of 2.2 mg of the unacetylated compound and 6.2 mg of the acetate are shown in Table 38. The C-21-$COCH_3$ signal was observed at 2.10 ppm. The acetate had two additional methyl signals at 1.98 and 2.02 ppm. The chemical shifts of the C-18 and C-19 methyl-hydrogens of the unacetylated compound and its diacetate, shown in Table 38, more closely resemble those of a steroid in the 5β series. Assuming that compound VI is a 5β-steroid and that

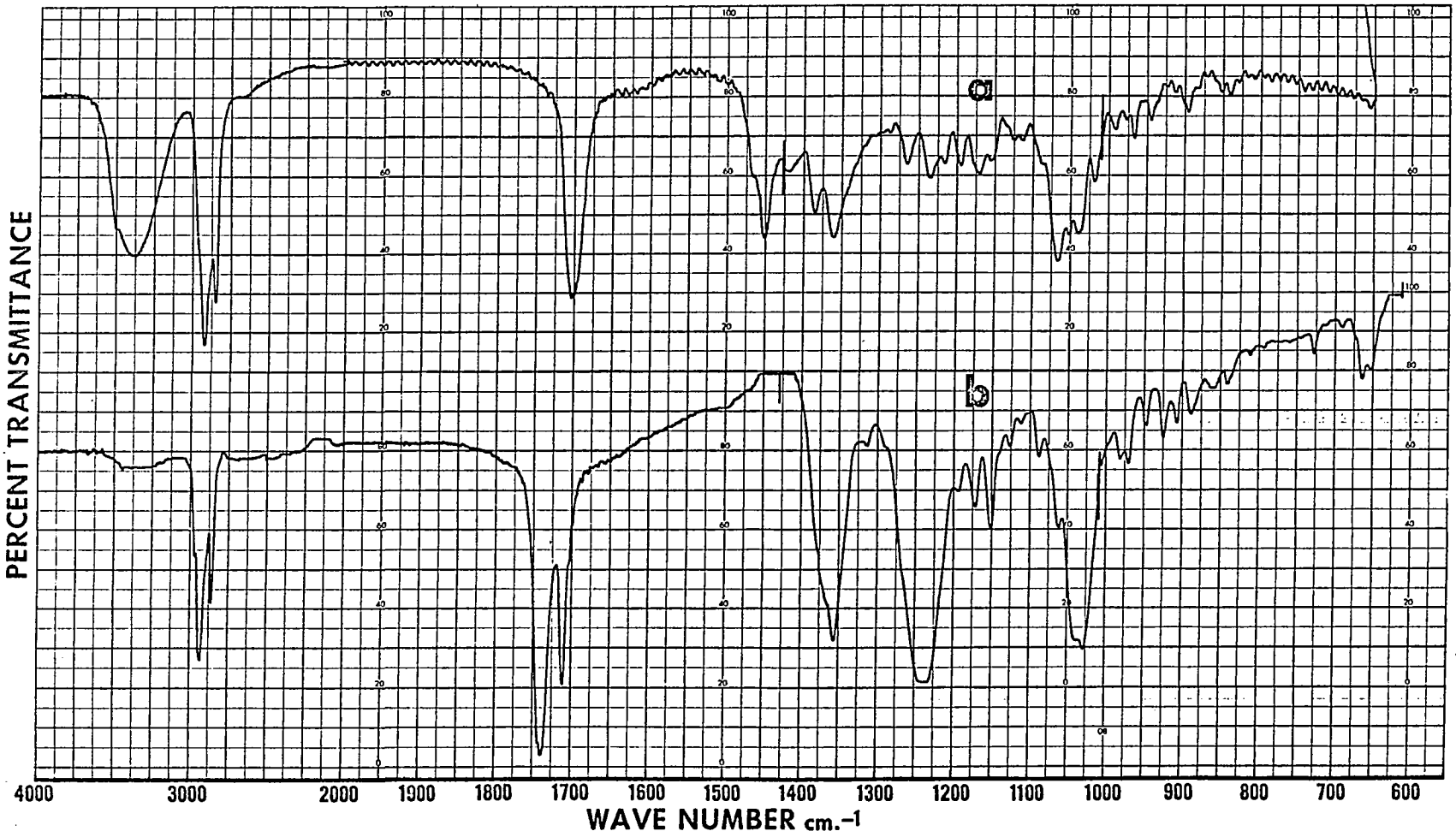


FIG. 10. (a) Infrared spectrum (KBr) of metabolite isolated from pool VI of neutral extract of urine.
(b) Infrared spectrum (CS_2) of the acetate of metabolite isolated from pool VI.

TABLE 38

Observed Chemical Shifts, δ(PPM), of Metabolite VI and its Diacetate and Calculated Shifts of 3α,15α-Dihydroxy-5β-pregnan-20-one and its Diacetate

Compound	Observed Chemical Shifts C-19	C-18	C-21-$COCH_3$	CH_3COO	C-3-H	Splitting Pattern of C-3-H
VI	0.93	0.64	2.10	-	3.6-4.1	axial
VI-Diacetate	0.94	0.67	2.10	1.98,2.02	4.5-4.9	axial

Compound	Calculated Chemical Shifts C-19	C-18
3α,15α-Dihydroxy-5β-pregnan-20-one	0.933	0.650
3α,15α-Diacetoxy-5β-pregnan-20-one	0.942	0.684

the acyl group at C-17 remains β, it is possible to calculate chemical shifts for the C-18 and C-19 methyls for different likely structures using Zurcher's Tables (45). The calculations for the 3α,15α-diol and the diacetate in the 5α,14α-series agree quite well (Table 38). The results for either 3α,15β-diol or 3β,15α-diol were sufficiently far off rendering it unlikely that any change in the configuration at C-15 had occurred. However, the calculations for the 3β,15α-diol were quite close to those obtained for VI but they did not fit as well as the results for the 3α,15α-diol. If the assignment 3α,15α-diol is correct, then the C-3 proton should be axial and hence the C-3 proton absorption should be broader due to two large axial-axial couplings, and should be shifted to a higher field. The NMR spectra of VI and its diacetate both show low-field signals which are quite broad and one is definitely shifted to higher field (3.6, 4.1 ppm in IV, and 4.5, 4.9 ppm in the diacetate), tending to confirm these assignments. These data indicated that the unknown VI, Table 36, Figure 7, was 3α,15α-dihydroxy-5β-pregnan-20-one. This conclusion is strengthened by the information obtained from the infrared spectra, the number of acylable hydroxy groups, and from the mass spectrum of the free compound (m/e = 334).

From Pool VIII, Figure 7, Table 36, a residue was obtained which was chromatographed on paper in system A for 4 days. Two main peaks of radioactive material VIIIA and VIIIB were obtained at average distances of 8.0 and 13.5 cm, respec-

tively. The eluate of VIIIA (4.45 mg and 1.50×10^5 dpm) was chromatographed on a 1 g silica gel column and the material eluted with 8% ethanol in methylene chloride gave a yellow residue which weighed 1.9 mg and contained 1.10×10^5 dpm. Attempts to crystallize this material were unsuccessful and because of the small amount present it could not be identified.

From fraction VIIIB an eluate was obtained which weighed 6.5 mg and contained 1.38×10^5 dpm. Chromatography on a 1 g silica gel column and elution with 8% ethanol in methylene chloride gave 1.5 mg of yellow residue containing 9.80×10^4 dpm which could not be crystallized. Due to insufficient amounts isolated, the metabolite present in VIIIB was not identified.

The material present in Pool IX (29.0 mg and 6.60×10^5 dpm) was chromatographed on two papers using system A for 4 days. One band of radioactive material was obtained with an average mobility of 12.2 cm which on elution yielded 14 mg of residue containing 6.40×10^5 dpm. Chromatography on a 2 g silica gel column and elution with 10% ethanol in methylene chloride gave a yellow oil which weighed 9.0 mg and contained 6.15×10^5 dpm. Crystallization from acetone-Skellysolve B afforded 1.6 mg of small plates: mp 211-214°C, specific activity 1.96×10^5 dpm/mg. Its infrared spectrum (Figure 11a) showed a strong hydroxyl band at 3250 cm^{-1} and no carbonyl bands indicating that both the Δ^4-3-keto and the 20-keto groups of 15α-hydroxyprogesterone had been reduced during the course of metabolism. An aliquot of the crystals (0.95 mg) was acetylated with ^{14}C-acetic anhydride, solution No. 2, and the product was chromatographed on a small alumina column. Elution with 40-50% benzene in

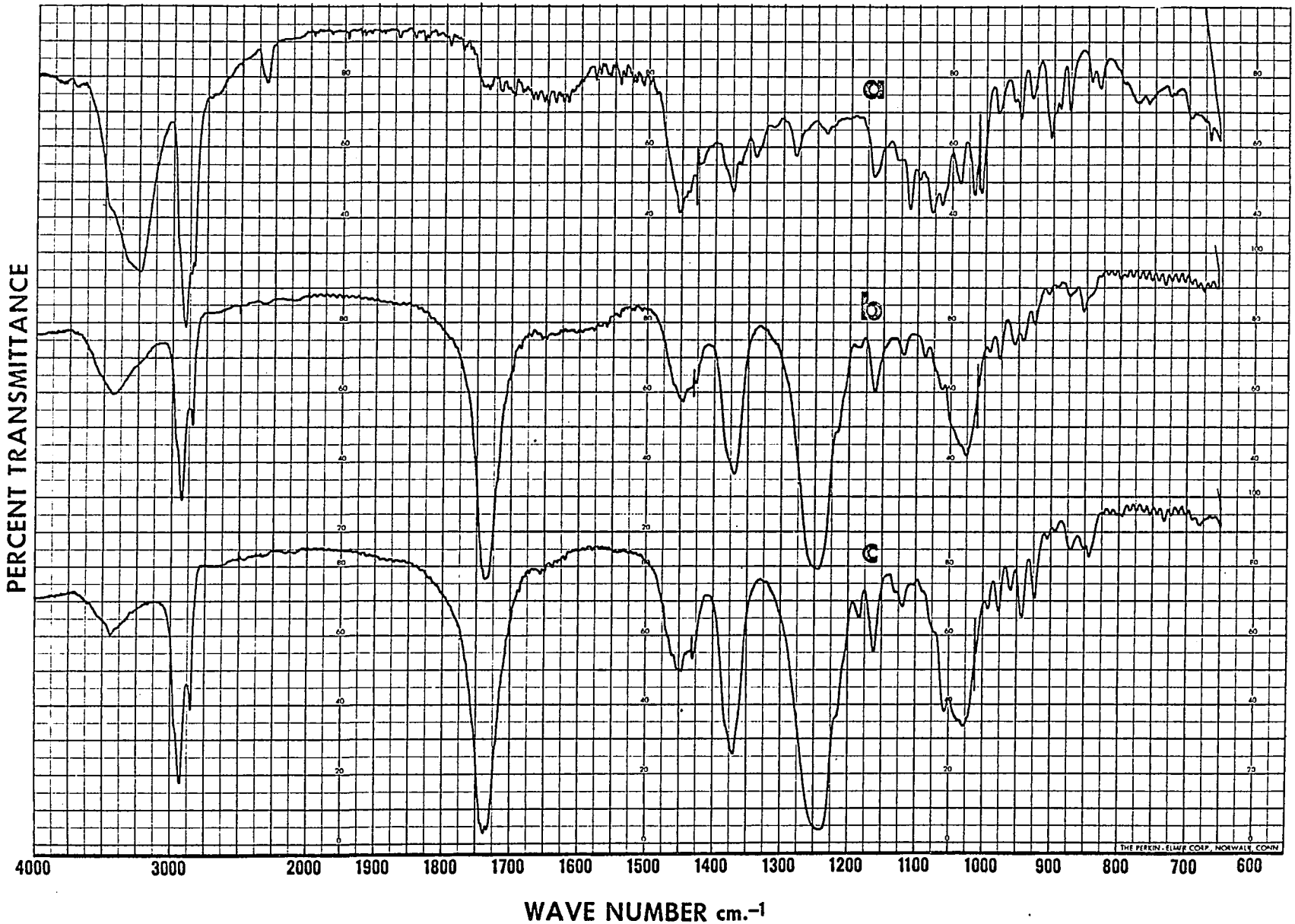


FIG. 11. (a) Infrared spectrum (KBr) of metabolite isolated from pool IX of neutral extract of urine.
(b) Infrared spectrum (CS_2) of the acetate of metabolite isolated from pool IX.
(c) Infrared spectrum (CS_2) of the acetate of the product obtained following $NaBH_4$ reduction of an aliquot of the metabolite isolated from pool V.

Skellysolve B gave 1.2 mg of oily material which had a specific activity of 5.52×10^4 dpm of ^{14}C/mg ($^3H/^{14}C$ = 3.1) indicating the presence of three acylable hydroxyl groups and its infrared spectrum (CS_2) is shown in Figure 11b. The spectrum showed the absence of a hydroxyl band at 3250 cm^{-1} indicating that all the hydroxyl groups on the molecule had been acetylated. The remainder of the triacetate of IX was used for NMR spectroscopy and the results are shown in Table 39. Instead of a methyl ketone at C-21 (2.10 ppm), the spectrum showed an absorption band at 1.21 ppm which can be attributed to a secondary hydroxyl group arising from the C-20 ketone which was reduced to an alcohol. The C-18 and C-19 methyl absorptions appeared at 0.72 and 0.80 ppm, respectively, corresponding to the absorptions observed with steroids having a 5α hydrogen. The pattern of absorption in the low-field at 4.95 ppm indicates that the proton at C-3 is equatorial. If these assignments are correct, then the possible structures which can be assigned to IX are 5α-pregnane-3α,15α,20α-triol or 5α,pregnane-3α,15α,20β-triol. The calculated positions of C-18 and C-19 methyl absorptions for 5α-pregnane-3α,15α,20α-triacetate and 5α-pregnane-3α,15α,20β-triacetate and the observed positions for the triacetate of IX are shown in Table 39. The absorption of the C-19 methyl (0.80 ppm) is in good agreement with the calculated values for both the 20α- and 20β-triol. The position of the signal of C-18 (0.72 ppm) is just between the two calculated values (0.703 and 0.753 ppm) and close enough to both that no decisive choice can be made.

TABLE 39

Observed Chemical Shifts, δ(PPM), of the Triacetate of IX and Calculated Chemical Shifts of 5α-Pregnane-3α,15α,20α-triacetate and 5α-Pregnane-3α,15α,20β-triacetate

	Observed Chemical Shifts					
Compound	C-19	C-18	C-21-CH_3CHOH	CH_3COO	C-3-H	Splitting Pattern of C-3-H
IX-Triacetate	0.80	0.72	1.21	1.99,2.04	4.95	equatorial

Compound	Calculated Chemical Shifts	
	C-19	C-18
5α-Pregnane-3α,15α,20α-triacetate	0.809	0.753
5α-Pregnane-3α,15α,20β-triacetate	0.809	0.703

Thus the question of whether IX has a 20α- or a 20β-hydroxyl group is not readily answered from the NMR data. When an aliquot (1.0 mg) of the diacetate of V (3α,15α-diacetoxy-5α-pregnan-20-one) was reduced with $NaBH_4$ and the product acetylated, the resulting triacetate had an infrared spectrum (CS_2) almost identical to that of IX-triacetate as shown in Figure 11c. Minor differences between the two spectra (Figure 11, spectra b and c) may be due to the presence of impurities in IX-triacetate since only small amounts (1.2 mg) of this compound were available and could not be crystallized. Thus the question of whether IX was a 20α- or a 20β-hydroxyl group could be answered by establishing the configuration of the C_{20}-hydroxyl resulting from the reduction of a 15α-hydroxylated-20-ketosteroid with $NaBH_4$.

Fieser and Fieser (46) summarized the molecular rotation (MD) data for nineteen pairs of 20-epimers of pregnanes and pointed out that the shift in MD on acetylation is positive for 20β-ols and negative for 20α-ols. An aliquot of 15α-acetoxyprogesterone (110 mg) was reduced with $NaBH_4$, oxidized with DDQ, and the product chromatographed on a 12 g alumina column. Elution with 0.5% ethanol in benzene yielded 105 mg of material which was crystallized from acetone-Skellysolve B to give 92 mg of large needles, mp 192-193°C. An aliquot of the crystals (50 mg) was acetylated with non-labeled acetic anhydride and the product chromatographed on a 6 g alumina column from which 53 mg of material was eluted with benzene-Skellysolve B (2:1).

Crystallization from acetone-Skellysolve B afforded 36 mg of small plates, mp 176-179°C. An aliquot (30 mg) of the 20-alcohol obtained after reduction of 15α-acetoxyprogesterone with $NaBH_4$ and oxidation with DDQ, and an aliquot (31 mg) of the 20-acetate, prepared as described above, were used for optical rotation studies (courtesy of Dr. G. Schilling, Ayerst Laboratories, Montreal). The optical rotations of progesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 20α-acetoxyprogesterone and 20β-acetoxyprogesterone were also measured. Optical rotation measurements were obtained using chloroform solutions. Table 40 shows the shifts in M_D on acetylation of 20α-dihydroprogesterone and 20β-dihydroprogesterone. The same correlation reported by Fieser and Fieser (46) was observed in that the ΔM_D of the 20α-epimer was slightly negative (-40) while the ΔM_D of the 20β-epimer was positive (+212°). The 20-alcohol obtained after reduction of 15α-acetoxyprogesterone with $NaBH_4$ and its 20-acetate (15α,20-diacetoxyprogesterone) had an ΔM_D of +179° (Table 40). These results provide evidence that $NaBH_4$ reduction of 15α-hydroxylated-20-ketosteroids results in the formation of 20β-alcohols. Further evidence to support this conclusion was obtained with 20β-hydroxysteroid dehydrogenase which reduces a 20-ketosteroid to the corresponding 20β-alcohol. On page 28, the preparation of 15α-acetoxy-20β-hydroxypregn-4-en-3-one by reduction of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase was described and it was indicated that the infrared spectrum (CS_2) of this material was identical to the spectrum of the material obtained after reduction of

15α-acetoxyprogesterone with $NaBH_4$ and oxidation with DDQ. These spectra are shown in Figure 12.

TABLE 40

Optical Rotation Data of 20-Hydroxylated Steroids and their 20-Acetates

Steroid	Molecular Rotation (M_D)	ΔM_D	
Progesterone	+635	0	
20α-Dihydroprogesterone	+329	-306	
20α-Acetoxyprogesterone	+323	-312	ΔM_D = -6
20β-Dihydroprogesterone	+272	-363	
20β-Acetoxyprogesterone	+484	-151	ΔM_D = +212
Product Obtained following $NaBH_4$ reduction and DDQ oxidation of 15α-Acetoxy-progesterone	+393	-242	
15α,20-Diacetoxyprogesterone*	+572	- 63	ΔM_D = +179

*The acetate of the product obtained following $NaBH_4$ reduction and DDQ oxidation of 15α-acetoxyprogesterone.

The results described above provide strong evidence that $NaBH_4$ reduction of a 15α-hydroxylated-20-ketosteroid results in the formation of the corresponding 20β-alcohol. Since reduction of V-diacetate (3α,15α-diacetoxy-5α-pregnan-20-one) and acetylation of the product gave material having an infrared spectrum identical (or almost identical) to the spectrum of IX-triacetate, metabolite IX was identified as 5α-pregnane-3α,15α,20β-triol and its mass spectrum (m/e = 336) is in agreement with this structure.

The residue of pool X, Figure 7, Table 36, was chromatographed on two Whatman No. 3MM papers in system A for 8 days. Two

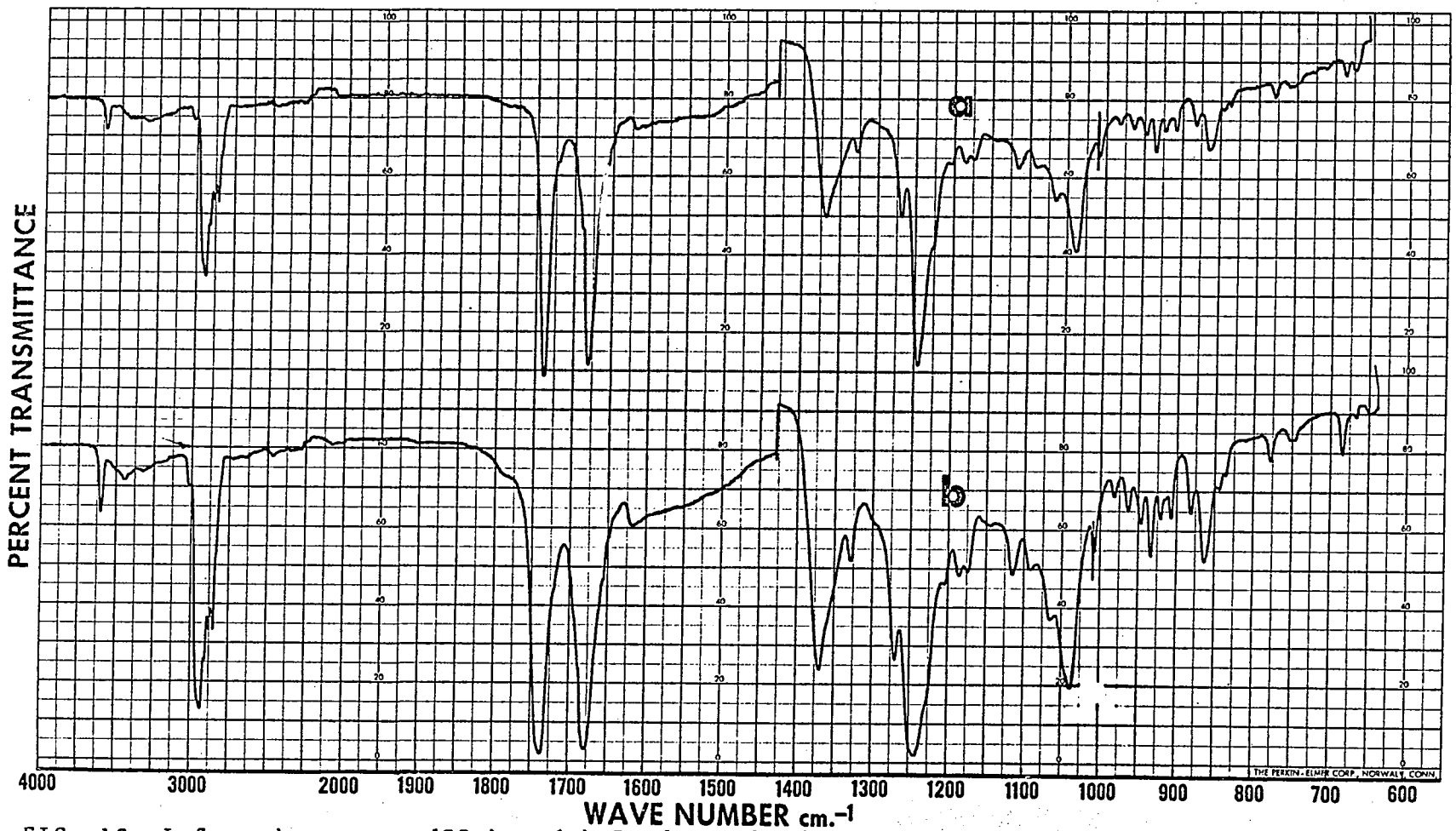


FIG. 12. Infrared spectra (CS_2). (a) Product obtained following $NaBH_4$ reduction and DDQ oxidation of 15α-acetoxyprogesterone.
(b) Product obtained following incubation of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase and β-DPNH.

major radioactive products, XA and XB, were obtained. The eluate from XA (18.5 mg and 2.80×10^5 dpm) was chromatographed on a 2 g silica gel column and elution with 11% ethanol in methylene chloride gave a residue which weighed 3.0 mg and contained 2.30×10^5 dpm. This material could not be crystallized and was not sufficiently pure and therefore could not be identified.

From XB a residue was obtained (25.0 mg and 7.15×10^5 dpm) which was chromatographed on a 3 g silica gel column. The material eluted with 12% ethanol in methylene chloride weighed 5.3 mg and contained 6.83×10^5 dpm. Attempts to crystallize this material were unsuccessful. Its infrared spectrum (KBr) showed a strong hydroxyl band at 3375 cm^{-1} and had no carbonyl bands. When an aliquot of VI (3α,15α-dihydroxy-5β-pregnan-20-one, 1.0 mg) was reduced with $NaBH_4$ and the product chromatographed on a small silica gel column, it was possible to obtain 0.9 mg of colorless oil in 12% ethanol in methylene chloride which had an infrared spectrum almost identical to that of XB. These results indicate that XB might be 5β-pregnane-3α,15α,20α-triol or 5β-pregnane-3α,15α,20β-triol.

In the study described it was possible to isolate and identify the following urinary steroids: 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3α,15α,20β-triol.

Experiment C-2. Metabolism of 15α-Hydroxyprogesterone by the Normal Male

This study was designed to obtain large amounts of the metabolites isolated in Experiment C-1 to be used as carriers for the identification of the urinary metabolites of 15α-hydroxyprogesterone after the intravenous injection of high specific activity (4-^{14}C)-15α-hydroxyprogesterone into a pregnant and a non-pregnant female which will be described in Experiments C-3 and C-4.

Four normal males volunteered for this study. Each subject was given 800 mg of 15α-hydroxyprogesterone by mouth, in 80 mg doses, contained in gelatin capsules, over a period of ten hours. A total of 3.25 g of 15α-hydroxyprogesterone was ingested by the four subjects. After the administration of the first dose urine was collected for four days. The urine collected from the four subjects was pooled and the steroid conjugates were hydrolyzed with Glusulase. The neutral extract obtained after hydrolysis weighed 3.5 g and it was chromatographed on a 250 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. The effluent from the column was collected in 20 ml fractions at a rate of 50-60 ml/hour. Since no radioactivity was used in this study, the fractions obtained from the column were pooled on the basis of visual examination of the tubes after they had been placed in a fume hood and the solvent evaporated. The mode of elution and the weight of the residue ob-

tained from each pool are shown in Table 41.

The material in Pool II, Table 41, was chromatographed on 30 TLC plates using system N. One ultraviolet-absorbing band with the mobility of 15α-hydroxyprogesterone was obtained. On elution it gave 190 mg of material which was crystallized from acetone to yield 145 mg of small plates. The crystals had a mp of 230-232°C and an infrared spectrum (KBr) identical to that of 15α-hydroxyprogesterone.

The residue obtained from Pool IV, Table 41, was chromatographed on 7 TLC plates using system N and one phosphomolybdic acid-positive spot was obtained having an R_f of 0.45. The material eluted weighed 95 mg and it was chromatographed on a 10 g alumina column. Elution with 1.5% ethanol in benzene gave 83 mg of material which was crystallized from acetone-Skellysolve B to give 45 mg of coarse plates: mp 192-195°C. Its infrared spectrum (KBr) showed a hydroxyl band at 3350 cm^{-1} and a carbonyl band at 1710 cm^{-1} corresponding to a 20-ketone (Figure 13). An aliquot of the crystals (40.0 mg) was combined with the mother liquor obtained after crystallization of IV and the mixture (78.0 mg) was acetylated with non-labeled acetic anhydride. The resulting acetate was chromatographed on an 8 g alumina column and elution with 50-60% benzene in Skellysolve B gave 86.0 mg of material which was crystallized from acetone-Skellysolve B to yield 66.0 mg of fine needles: mp 152-155°C. The infrared spectrum of the cyrstals (Figure 13) did not have a hydroxyl band indicating that all the hydroxyl groups of the molecule had

TABLE 41

Elution of Material from the Initial Silica Gel Column Based on Visual Examination of the Fractions Collected

Pool	Fraction No.	% Ethanol in Methylene Chloride	Weight (mg)	Remarks
I	1-173	2	120	-
II	174-230	3	280	Crystalline material
III	231-280	3	45	-
IV	281-320	3	110	Crystalline material
V	321-440	4	230	" "
VI	441-530	4-6	460	" "
VII	531-785	6-8	80	-
VIII	786-820	10	42	Crystalline material
IX	821-880	10	32	-
X	881-395	12	110	Crystalline material
XI	936-1096	12	90	-

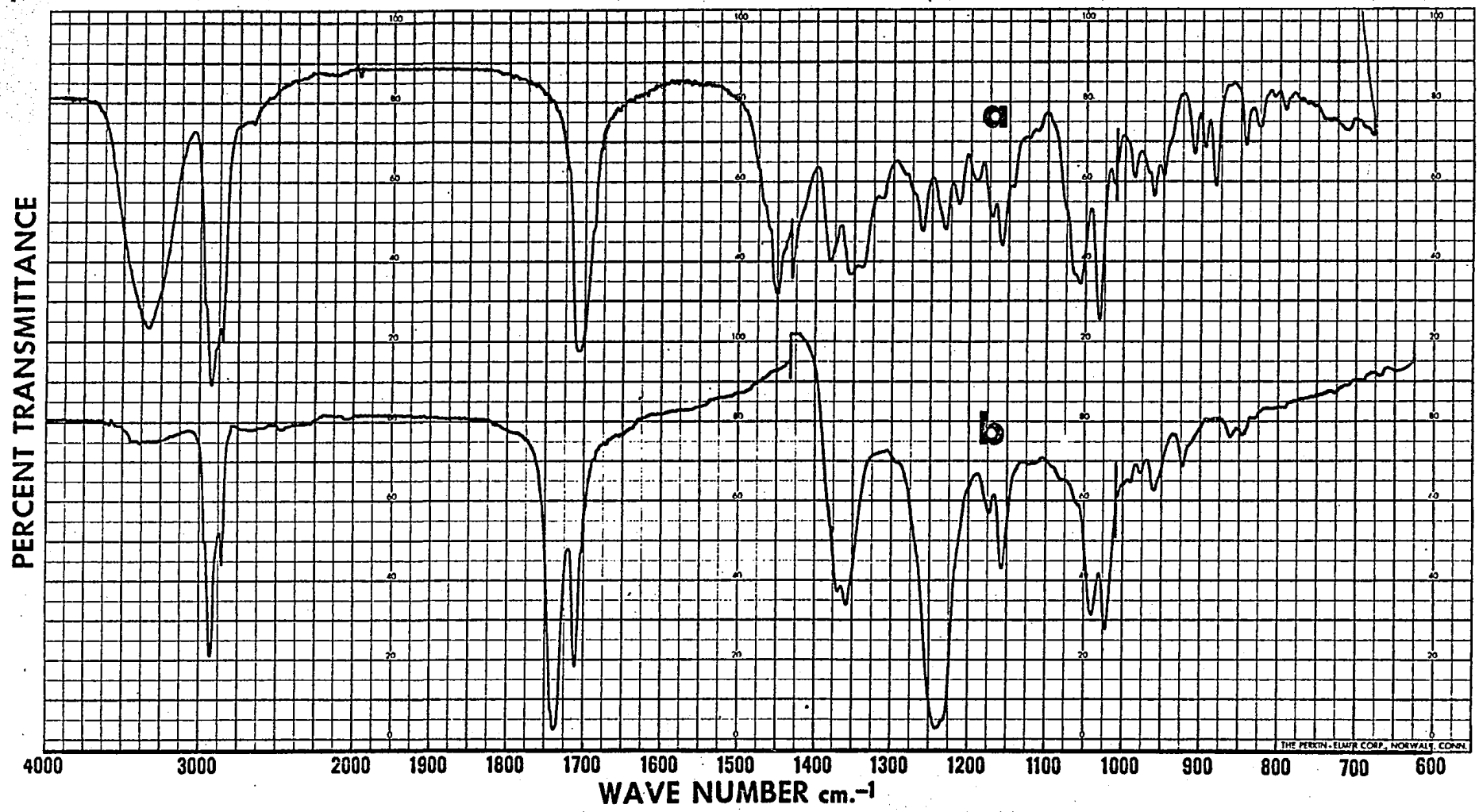


FIG. 13. (a) Infrared spectrum (KBr) of metabolite isolated from pool IV of neutral extract of urine.
(b) Infrared spectrum (CS_2) of the acetate of metabolite isolated from pool IV.

been acetylated. Another aliquot of IV (2.0 mg) was acetylated with ^{14}C-acetic anhydride solution No. 2. After chromatography on a small alumina column the acetate had a specific activity of 3.80×10^4 dpm ^{14}C/mg. Since the specific activity of acetic anhydride, solution No. 2, was 2.30×10^4 dpm of ^{14}C/mg of deoxycorticosterone acetate (DOCA), a specific activity of 3.80×10^4 dpm of ^{14}C/mg indicated that IV had two acylable hydroxyl groups (specific activity of IV/specific activity of DOCA = 1.65). The results obtained from NMR analysis of the unacetylated compound and its diacetate are shown in Table 42. The C-21-$COCH_3$ signal was observed at 2.12 ppm. The acetate had two additional signals at 2.00 and 2.05 ppm which indicated that the Δ^4-3-keto system had been reduced without affecting the 20-keto group, a finding confirmed by the infrared spectrum of IV. The chemical shifts of the C-18 and C-19 methyl-hydrogens of the unacetylated compound and of the diacetate (Table 42) correspond closely with those calculated for 5β-steroids. Assuming that compound IV is a 5β-steroid and that the acyl group at C-17 remains β, it is possible to calculate positions for C-18 and C-19 absorptions for different likely structures using Zurcher's Tables (45). The results for the 3β,15α-diol and its diacetate in the 5β,14α-series agree quite well as is shown in Table 42. If the assignment 3β,15α-diol is correct, then the hydrogen at C_3 should be equatorial and hence the C_3-H absorption should be sharper due to both axial-equatorial and equatorial-equatorial coupling being of medium size, and to

TABLE 42

Observed Chemical Shifts, δ(PPM), of Metabolite IV and its Diacetate and Calculated Chemical Shifts of 3β,15α-Dihydroxy-5β-pregnan-20-one and its Diacetate

	Observed Chemical Shifts					
Compound	C-19	C-18	C-21-$COCH_3$	CH_3COO	C03-H	Splitting Pattern of C-3-H
IV	0.99	0.64	2.12	-	3.9-4.2	equatorial
IV-diacetate	0.98	0.68	2.12	2.00, 2.05	4.8-5.2	equatorial

Compound	Calculated Chemical Shifts C-19	C-18
3β,15α-Dihydroxy-5β-pregnan-20-one	0.975	0.650
3β,15α-Diacetoxy-5β-pregnan-20-one	0.975	0.684

lower field. The NMR spectrum of the unacetylated compound had the two low-field protons superimposed at 3.9-4.2, and that of the acetate at 4.8-5.2; but the pattern had the appearance of a sharper signal atop of a broader one. All these results together permit the assignment of the structure 3β,15α-dihydroxy-5β-pregnan-20-one to the metabolite in pool IV. The mass spectrum of IV (m/e = 334) is in agreement with this structure.

The residue of pool V, Table 41, was chromatographed on 15 thin layer plates using system N and one major band was observed (R_f = 0.40) when 0.5 cm strips of the plates were sprayed with phosphomolybdic acid. On elution 179 mg of colorless oil was obtained which had an infrared spectrum (KBr) identical to that of 3α,15α-dihydroxy-5α-pregnan-20-one. It was acetylated with non-labeled acetic anhydride and the product chromatographed on a 20 g alumina column. The material eluted with 50-60% benzene in Skellysolve B (182.0 mg) was crystallized from acetone-Skellysolve B to yield 86.5 mg of fine needles which had an mp of 142-144°C and an infrared spectrum (CS_2) identical to that of 3α,15α-diacetoxy-5α-pregnan-20-one.

Pool VI, Table 41, yielded a residue which weighed 460 mg and it was chromatographed on 28 thin layer plates in system N. The area corresponding to 3α,15α-dihydroxy-5β-pregnan-20-one was eluted to give 402 mg of crystalline material which had an infrared spectrum (KBr) identical to that of 3α,15α-di-

hydroxy-5β-pregnan-20-one. An aliquot of this material (201 mg) was acetylated with non-labeled acetic anhydride and the product chromatographed on a 50 g alumina column. The material eluted with 50-60% benzene in Skellysolve B (236 mg) was crystallized from acetone-Skellysolve B to give 178 mg of small plates: mp 164-167°C. Its infrared spectrum (CS_2) was identical to that of 3α,15α-diacetoxy-5β-pregnan-20-one.

The residue of pool VIII, Table 41, was chromatographed on three thin layer plates using system K, and the area corresponding to 5α-pregnane-3α,15α,20β-triol was eluted to give 15.0 mg of oily material. Crystallization from acetone-Skellysolve B gave 9.0 mg of small plates: mp 211-214°C. Its infrared spectrum was identical to that of 5α-pregnane-3α,15α,20β-triol. Because only a small amount of 5α-pregnane-3α,15α,20β-triol was isolated, a large quantity of this compound was prepared by the reduction of 3α,15α-dihydroxy-5α-pregnan-20-one with $NaBH_4$. The mother liquor obtained from the crystallization of 3α,15α-dihydroxy-5α-pregnan-20-one (Pool V) was reduced wtih $NaBH_4$ and after purification on a 10 g silica gel column 83.0 mg of oily material was eluted (with 10% ethanol in methylene chloride) whose infrared spectrum (KBr) was identical to that of 5α-pregnane-3α,15α,20β-triol. This material was combined with VIII and the mixture (92.0 mg) was acetylated and purified on a 10 g alumina column. Elution with 40% benzene in Skellysolve B gave 115.0 mg of colorless oil which was crystallized from ace-

tone-Skellysolve B to yield 76.0 mg of small plates: mp 134-137°C. Its infrared spectrum (CS_2) was identical to that of 5α-pregnane-3α,15α,20β-triacetate.

From pool X, Table 41, a residue was obtained which weighed 110 mg. It was chromatographed on eight thin layer plates in system K and one major area was observed with phosphomolybdic acid. On elution it gave 54.1 mg of residue which was chromatographed on a 5 g silica gel column. Elution with 12% ethanol in methylene chloride yielded 18.5 mg of crystalline material whose infrared spectrum (KBr) was identical to that of compound XB isolated in Experiment C-1 from Pool X, Figure 7, Table 36. The infrared spectrum of the material in X was again very similar to the spectrum of the compound obtained on reduction of 3α,15α-dihydroxy-5β-pregnan-20-one with $NaBH_4$ (Figure 14). Acetylation of an aliquot of X (12 mg) and purification of the acetate on a small alumina column gave 8.0 mg of crystalline material in benzene-Skellysolve B (2:3) which was crystallized from acetone-Skellysolve B to yield 4.5 mg of small plates: mp 206-208°C. Its infrared spectrum (CS_2) was almost identical to the spectrum of the compound obtained after acetylation of the $NaBH_4$-reduction product of 3α,15α-dihydroxy-5β-pregnan-20-one (Figure 15). The only difference between the two spectra (a and b, Figure 15) is an extra band in spectrum a at 1145 cm^{-1} which might be due to the presence of impurities. The material isolated from X was not further purified because of the insufficient amount available.

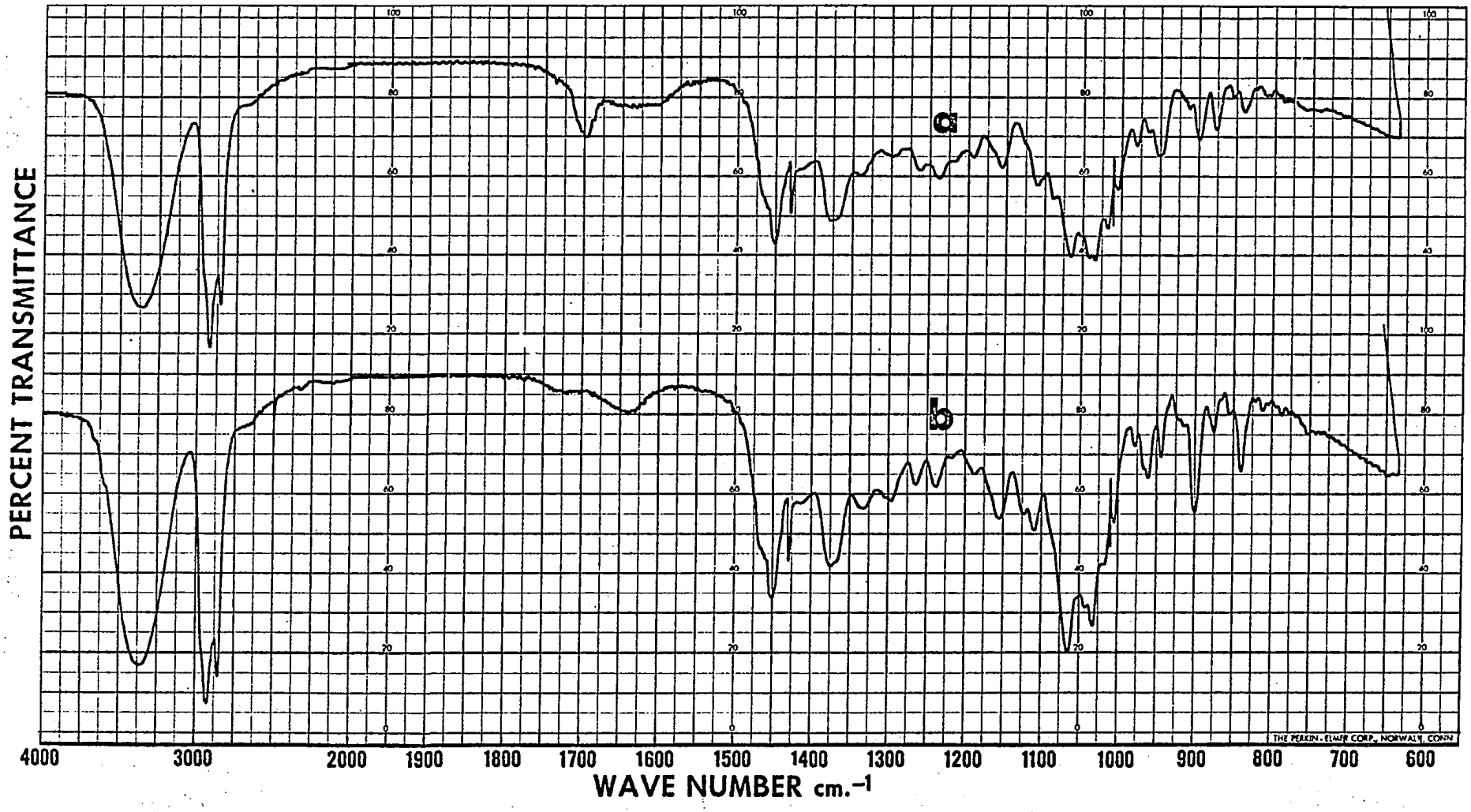


FIG. 14. Infrared spectra (KBr). (a) Metabolite isolated from pool X of neutral extract of urine.
(b) Product obtained following $NaBH_4$ reduction of 3α,15α-dihydroxy-5β-pregnan-20-one.

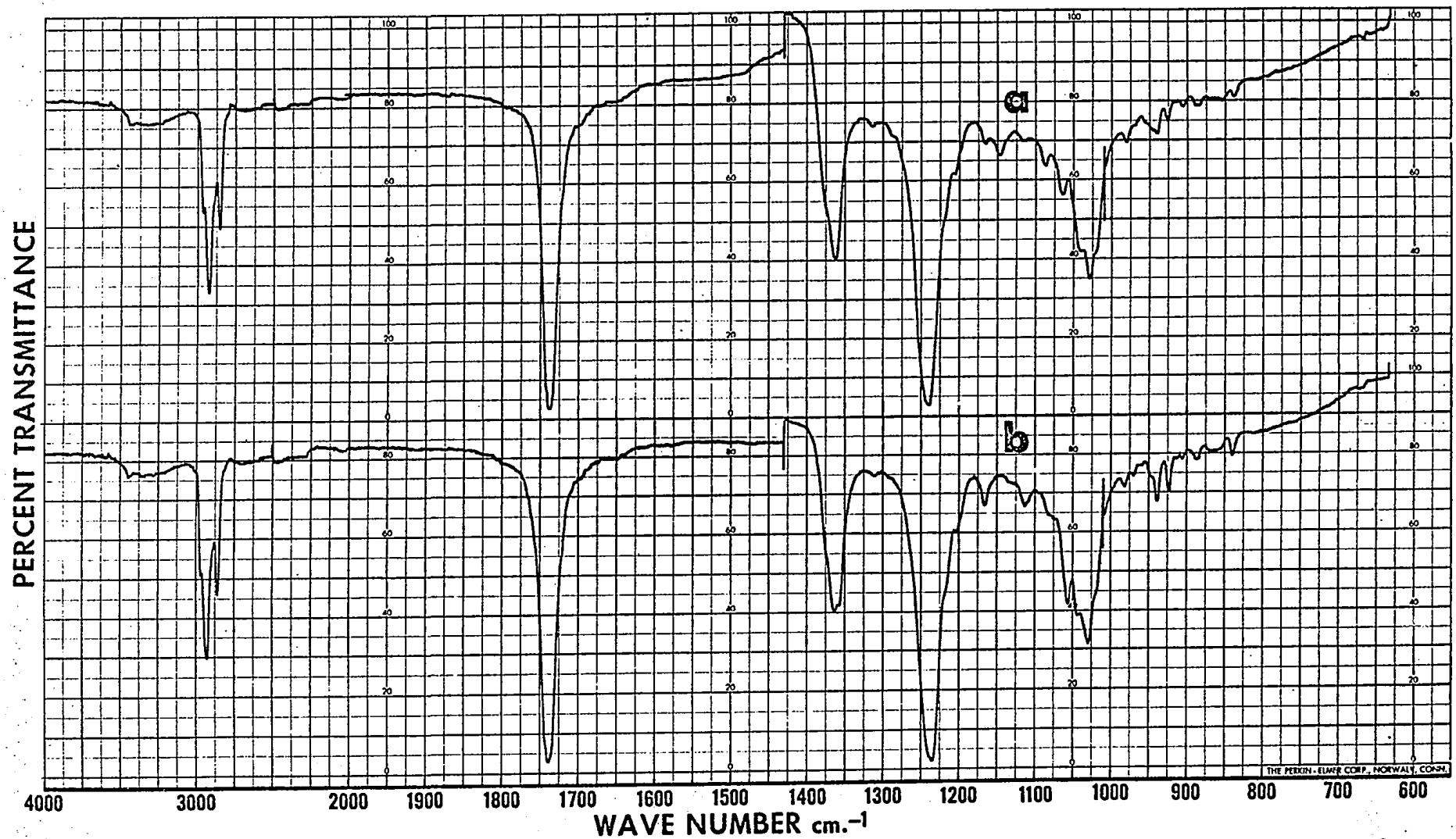


FIG. 15. Infrared spectra (CS_2). (a) Acetate of the metabolite isolated from pool X of neutral extract of urine.

(b) Acetate of the product obtained following $NaBH_4$ reduction of 3α,15α-dihydroxy-5β-pregnan-20-one.

An aliquot of X-triacetate was used for NMR spectroscopy and the results are shown in Table 43. Instead of a methyl ketone at C-21 (2.10 ppm) the spectrum showed an absorption band at 1.18 and 1.24 ppm which can be attributed to a secondary hydroxyl group arising from the C-20 ketone which was reduced to an alcohol. The C-18 and C-19 methyl absorptions appeared at 0.71 and 0.93 ppm, respectively, corresponding to the absorptions observed with steroids having a 5β-hydrogen. The pattern of absorption in the low-field at 4.7-5.0 ppm indicates that the proton at C-3 is axial. If these assignments are correct then the possible structures which can be assigned to X are, 5β-pregnane-3α,15α,20α-triol or 5β-pregnane-3α,15α,20β-triol. The calculated positions of C-18 and C-19 methyl absorptions for 5β-pregnane-3α,15α,20α-triacetate and 5β-pregnane-3α,15α, 20β-triacetate and the observed positions for the triacetate of X are shown in Table 43. The absorption of the C-19 methyl is in good agreement with the calculated values for both the 20α- and 20β-triol. The position of the signal of C-18 (0.71 ppm) is just between the two calculated values (0.734 and 0.684 ppm) and close enough to both that no decisive choice can be made. However, it was mentioned earlier that the infrared spectra of X-triacetate and of the triacetate of the material obtained after reduction of 3α,15α-dihydroxy-5β-pregnan-20-one with $NaBH_4$ were identical (or almost identical, see Figure 15). It was also established in Experiment C-1, Page 121, that $NaBH_4$ reduction of a 15α-hydroxylated-20-ketosteroid results in the formation

TABLE 43

Observed Chemical Shifts, δ(PPM), of the Triacetate of X and Calculated Chemical Shifts of 5β-pregnane-3α,15α,20α-triacetate and 5β-pregnane-3α,15α,20β-triacetate

Compound	C-19	C-18	C-21-CH_3CHOH	CH_3COO	C-3-H	Splitting Pattern of C-3-H
X-Triacetate	0.93	0.71	1.18,1.24	1.99,2.02	4.7-5.0	axial

Compound	Calculated Chemical Shifts C-19	C-18
5β-Pregnane-3α,15α,20α-triacetate	0.942	0.734
5β-Pregnane-3α,15α,20β-triacetate	0.942	0.684

of a 20β-alcohol. Therefore the structure 5β-pregnane-3α,15α, 20β-triol was assigned to metabolite X and the mass spectrum of X (m/e = 336) is in agreement with this structure.

In this study it was possible to isolate and identify the following urinary steroids: 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,15α,20β-triol, 3β,15α-dihydroxy-5β-pregnan-20-one and 5β-pregnane-3α,15α,20β-triol. The first four metabolites were also isolated in Experiment C-3. However in this study two additional metabolites of 15α-hydroxyprogesterone, namely, 3β,15α-dihydroxy-5β-pregnan-20-one and 5β-pregnane-3α,15α,20β-triol, were isolated and identified.

Experiment C-3. Metabolism of 15α-Hydroxyprogesterone by the Pregnant Female

A normal subject in the 38th week of pregnancy was injected wtih 4.90×10^6 dpm of (4-^{14}C)-15α-hydroxyprogesterone contained in 13.4 μg. Urine was collected for six days and the conjugates present in each day's urine were first solvolyzed and then hydrolyzed with β-glucuronidase. The total radioactivity recovered in each day's urine and the amount released by solvolysis and by β-glucuronidase hydrolysis are shown in Table 44. Since virtually all of the radioactivity was present in the extracts from the first four days' urine, the extracts from the urine collected in days 5 and 6 were not processed.

The sulfate fraction weighed 450 mg and contained 3.36×10^5 dpm. It was chromatographed on a 50 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described and the radioactivity in the eluted fractions is shown in Figure 16. The radioactive material present in each of the pools eluted is shown in Table 45.

Pool IV, Figure 16, Table 45, gave a residue which was chromatographed on two Whatman No. 3MM papers in system A for 15 hours. One major radioactive zone corresponding in mobility to 3β,15α-dihydroxy-5β-pregnan-20-one was obtained which was eluted to give 7.5 mg of residue containing 7.20×10^4 dpm. It was chromatographed on paper in system L for 8 hours and a single peak of radioactivity with the mobility of 3β,15α-dihydroxy-5β-pregnan-

TABLE 44

Recovery of Radioactivity in the Urine and the Extracts Obtained After Hydrolysis of Conjugates by Solvolysis and by β-Glucuronidase

Day	Crude Urine (dpm)	% Recovery of Injected Dose	Sulfates	% Recovery of Injected Dose	Glucosiduronates	% Recovery of Injected Dose
1	3.65×10^6	74.5	3.04×10^5	6.2	3.12×10^6	63.8
2	1.25×10^5	2.6	1.91×10^4	0.4	9.50×10^4	1.9
3	2.50×10^4	0.5	6.90×10^3	0.1	1.47×10^4	0.3
4	1.30×10^4	0.3	5.81×10^3	0.1	6.00×10^3	0.1
5	-	-				
6	-	-				
Total	3.81×10^6	77.9	3.36×10^5	6.8	3.24×10^6	66.1

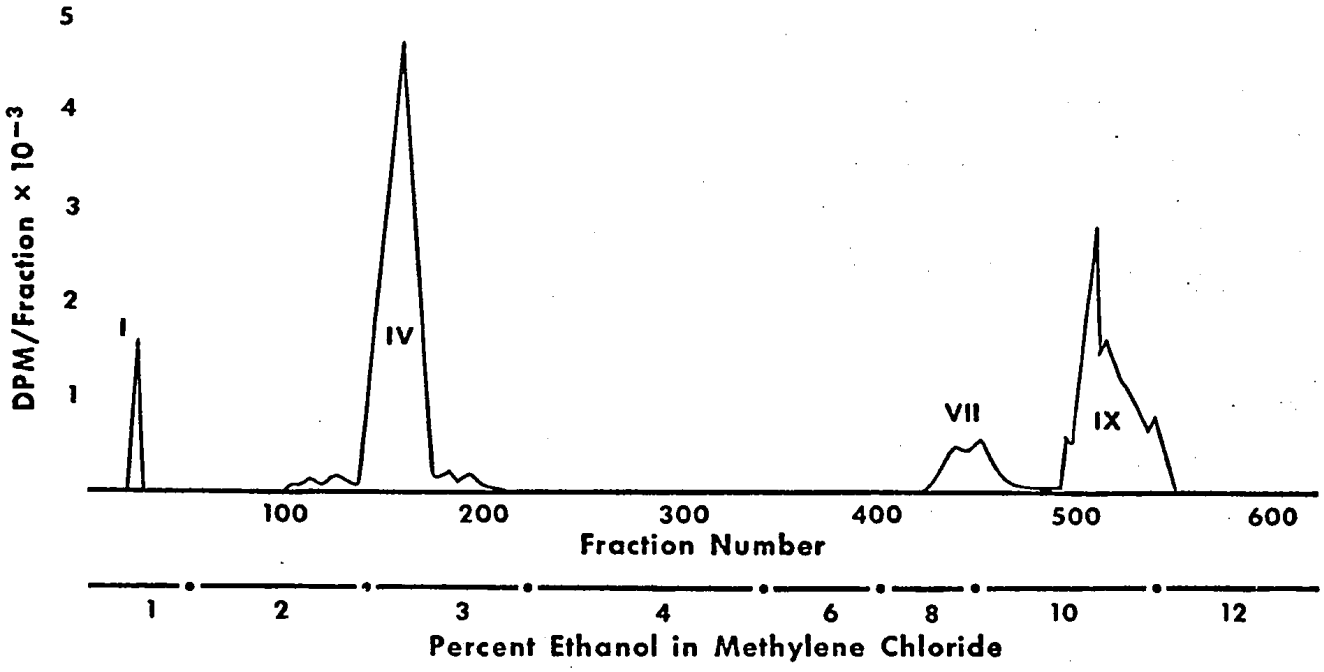


FIG. 16. Silica gel column chromatography of the sulfate fraction of urine following the administration of (4-^{14}C)-15α-hydroxyprogesterone to a pregnant female.

TABLE 45

Weight and Radioactivity Eluted from the Initial Silica Gel Column after Chromatography of the Extract from the Sulfate Fraction of Pregnancy Urine.

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	15-22	6.0	8.00×10^3
II	23-100	69.0	5.20×10^3
III	101-137	23.5	6.50×10^3
IV	138-176	35.6	8.70×10^4
V	177-210	21.0	7.50×10^3
IV	211-423	62.0	2.30×10^4
VII	424-474	24.3	1.25×10^4
VIII	475-493	12.7	5.10×10^3
IX	494-550	18.0	7.53×10^4
X	551-600	21.0	9.20×10^3

-20-one was obtained. After elution and chromatography on a small silica gel column, a residue (1.1 mg and 6.90×10^4 dpm) was eluted with 3% ethanol in methylene chloride. This material was acetylated with ^{3}H-acetic anhydride, Batch No. 5, and then mixed with 32.0 mg of carrier 3β,15α-diacetoxy-5β-pregnan-20-one. The mixture was chromatographed on a 4 g alumina column to yield 4.80×10^4 dpm of ^{14}C and 30.5 mg of material which was crystallized to constant specific activity and constant $^3H/^{14}C$ ratio as shown in Table 46. The crystals and mother liquor from the fourth crystallization were combined (12.5 mg) and the mixture was reduced with $NaBH_4$. Chromatography of the pro-

TABLE 46

Proof of Radiochemical Purity of 3β,15α-Dihydroxy-5β-pregnan-20-one Isolated from the sulfate Fraction of Pregnancy Urine

Crystallization	Crystals	Specific Activity (dpm ^3H/mgx10^3) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3β,15α-Diacetoxy-5β-pregnan-20-one				
1	19.3	12.2	80.8	50.5
2	15.7	9.7	43.9	28.3
3	13.9	8.6	19.8	12.4
4	13.6	8.3	14.0	8.7
3β,15α-Diacetoxy-5β-pregnan-20β-ol				
1	13.6	8.5	13.4	8.7
2	13.4	8.4	12.9	8.5
Calculated*	13.3			

*The fourth crystals and mother liquor of the diacetate were reduced with $NaBH_4$ and the product was chromatographed on a small alumina column to give 11.0 mg of 3β,15α-diacetoxy-5β-pregnan-20β-ol containing $1.47x10^5$ dpm of ^3H and $1.75x10^4$ dpm of ^{14}C.

duct on a 2 g alumina column and elution with benzene-Skellysolve B (1:1) yielded 11 mg of material containing $1.47x10^5$ dpm of ^3H and $1.75x10^4$ dpm of ^{14}C which was crystallized to constant specific activity and constant $^3H/^{14}C$ ratio as shown in Table 46. From the data shown in this table it was possible to assign the structure 3β,15α-dihydroxy-5β-pregnan-20-one to the metabolite in pool IV. The specific activity of this

metabolite was calculated to be 8.66×10^3 dpm/µg. The residues in all the other pools shown in Table 45 were not processed.

The glucosiduronate fraction (978 mg and 3.24×10^6 dpm) was chromatographed on a 100 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. In Figure 17 is shown a plot of the radioactivity eluted from which the different pools shown in Table 47 were obtained. Pools I, II, V, VII, VIII and IX contained material which could not be identified.

The residue of Pool III, Table 47, Figure 17, was chromatographed on two Whatman No. 3MM papers in system A for 10 hours. One peak of radioactive material was obtained having the same mobility as 15α-hydroxyprogesterone. On elution 8.5 mg of residue was obtained containing 1.25×10^6 dpm. This material was rechromatographed on one paper in system B for 5 days and one symmetrical zone of radioactivity was obtained corresponding in mobility to 15α-hydroxyprogesterone. The material eluted from the paper weighed 1.2 mg and contained 1.13×10^6 dpm. An aliquot of this material (2.82×10^5 dpm) was acetylated with ^{3}H-acetic anhydride, Batch No. 4, and the product mixed with 50.0 mg of carrier 15α-acetoxyprogesterone. The mixture was chromatographed on a 5 g alumina column and the residue obtained by elution with 80% benzene in Skellysolve B was crystallized to constant specific activity. The crystallization process was repeated on the derivative formed after $NaBH_4$ reduction followed by DDQ oxidation and the specific activities obtained are shown

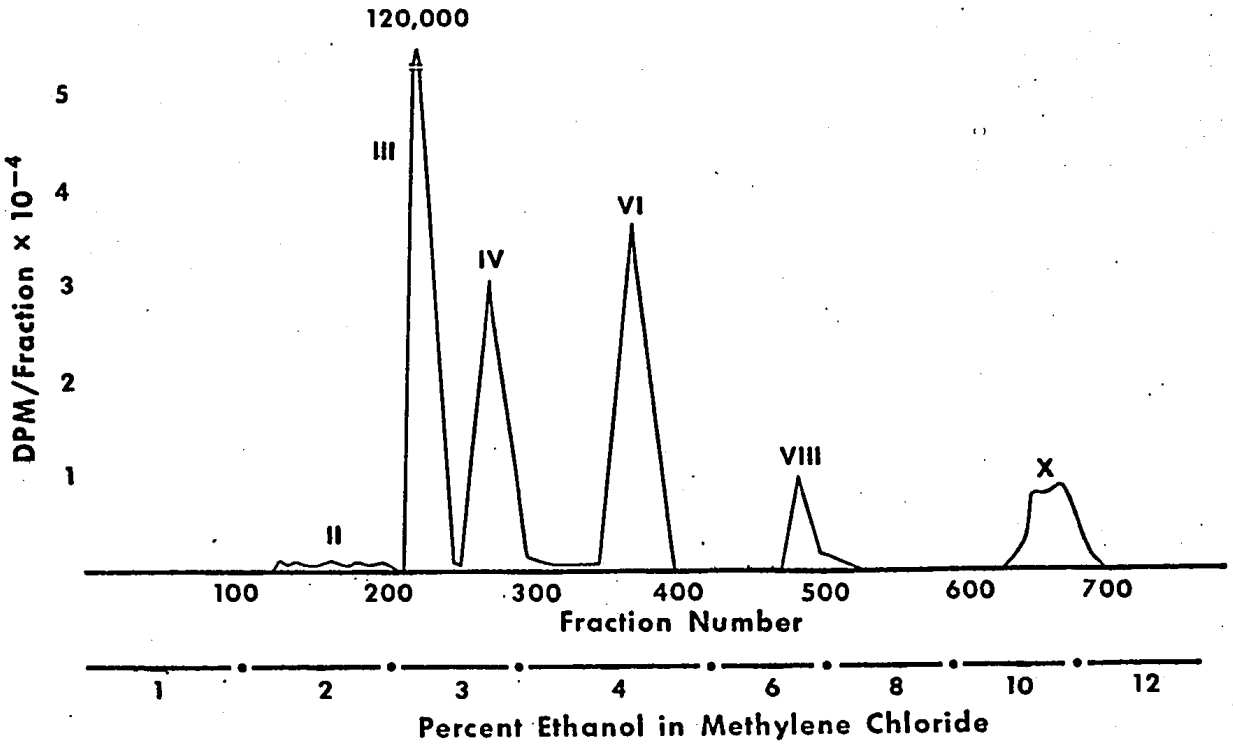


FIG. 17. Silica gel column chromatography of the glucosiduronate fraction of urine following the administration of (4-^{14}C)-15α-hydroxyprogesterone to a pregnant female.

TABLE 48

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated from the Glucosiduronate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mgx10^3) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
15α-Acetoxyprogesterone*				
1	29.0	5.7	100.5	20.8
2	16.9	3.3	32.5	6.3
3	12.3	2.4	55.5	10.9
4	12.0	2.3	12.4	2.4
15α-Acetoxy-20β-hydroxypregn-4-en-3-one				
1	10.7	2.4	10.8	2.4
2	10.6	2.4	10.7	2.4
Calculated**	11.0			

*After purification of the residue in pool III, an aliquot containing $2.82x10^5$ dpm was acetylated with ^3H-acetic anhydride, Batch No. 4, and mixed with 50.0 mg of carrier 15α-acetoxyprogesterone. The mixture was chromatographed on an alumina column to yield 50.0 mg of colorless oil containing $2.62x10^5$ dpm of ^{14}C.

**The fourth crystals and mother liquor were combined (19.8 mg), reduced with $NaBH_4$, and the product was oxidized with the DDQ reagent and then chromatographed on alumina to yield 12.5 mg of 15α-acetoxy-20β-hydroxypregn-4-en-3-one containing $1.37x10^5$ dpm of ^3H and $5.73x10^4$ dpm of ^{14}C.

in Table 48. From the data shown in this table it was possible to assign the structure 15α-hydroxyprogesterone to the metabolite in pool III. The specific activity of the isolated 15α-hydroxyprogesterone was calculated to be $1.56x10^4$ dpm/μg.

Residue IV, Figure 17, Table 47, was chromatographed on

four papers in system A for 24 hours and one main radioactive peak was obtained at an average distance of 15.5 cm, which corresponded to the mobility of 3α,15α-dihydroxy-5α-pregnan-20-one run alongside. This material was eluted (14.5 mg and 2.45×10^5 dpm) from the paper and it was rechromatographed on two papers in system L for 8 hours. A single peak of radioactive material was observed having the same mobility as 3α,15α-dihydroxy-5α-pregnan-20-one. Elution of this material from the papers gave 5.6 mg of residue containing 2.30×10^5 dpm which was chromatographed on a small silica gel column from which 2.0 mg of colorless oil was eluted containing 2.10×10^5 dpm. An aliquot of this material (3.50×10^4 dpm) was acetylated with ^{3}H-acetic anhydride Batch No. 4, mixed with carrier 3α,15α-diacetoxy-5α-pregnan-20-one and the mixture was chromatographed on a small alumina column. The residue obtained after elution with 50% benzene in Skellysolve B was crystallized to constant specific activity and the process was repeated after $NaBH_4$ reduction as shown in Table 49. From the data shown in this table it was possible to assign the structure 3α,15α-dihydroxy-5α-pregnan-20-one to the metabolite in pool IV. The specific activity of this metabolite was calculated to be 7.90×10^3 dpm/μg.

From pool VI, Figure 17, Table 47, a residue was obtained which was chromatographed on four Whatman, No. 3MM, papers in system A for 24 hours. One radioactive zone was obtained having the same mobility as 3α,15α-dihydroxy-5β-pregnan-20-one. The eluate (19.8 mg and 5.74×10^5 dpm) was rechromatographed on two

TABLE 49

Proof of Radiochemical Purity of 3α,15α-Dihydroxy-5α-pregnan-20-one Isolated from the Glucosiduronate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mgx10^3) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3α,15α-Diacetoxy-5α-pregnan-20-one*				
1	14.8	18.0	85.0	100.0
2	9.2	11.0	44.5	55.6
3	7.7	9.5	25.0	30.5
4	7.5	9.2	7.7	9.5
3α,15α-Diacetoxy-5α-pregnan-20β-ol				
1	7.4	9.3	7.7	9.4
2	7.6	9.1	7.4	9.2
Calculated**	7.5			

*An aliquot of the purified residue in pool IV containing $3.50x10^4$ dpm was acetylated with ^3H-acetic anhydride, Batch No. 4, mixed with 28.9 mg of carrier 3α,15α-diacetoxy-5α-pregnan-20-one and the diacetate chromatographed on alumina prior to crystallization.

**The fourth crystals and mother liquor of the diacetate were combined (13.0 mg) and the mixture was reduced with $NaBH_4$. The product was chromatographed on an alumina column from which 9.0 mg of material were eluted containing $6.5x10^4$ dpm of ^3H and $7.5x10^3$ dpm of ^{14}C.

papers in system L for 8 hours and one symmetrical peak of radioactivity was observed at an average distance of 20.5 cm, corresponding to the mobility of 3α,15α-dihydroxy-5β-pregnan-20-one run alongside. Elution from the paper yielded 7.5 mg of

residue containing 4.90×10^5 dpm. This material was chromatographed on a small silica gel column and elution with 4% ethanol in methylene chloride gave 3.5 mg of oily material containing 4.50×10^5 dpm. An aliquot of this material containing 4.50×10^4 dpm was acetylated with ^{3}H-acetic anhydride, Batch No. 4, and the product was mixed with 51.0 mg of carrier 3α,15α-diacetoxy-5β-pregnan-20-one. The mixture was chromatographed on a small alumina column and then crystallized. Constant specific activity was achieved after the fourth crystallization and again after $NaBH_4$ reduction as shown in Table 50. The data shown in this table permit the assignment of the structure 3α,15α-dihydroxy-5β-pregnan-20-one to the metabolite in pool VI. The specific activity of this metabolite was calculated to be 9.70×10^3 dpm/μg.

Pool X, Figure 17, Table 47, was chromatographed on two papers in system A for 8 days. Two main peaks of radioactive material, XA and XB, were obtained at average distances of 6.0 and 13.5 cm, respectively. The material in peak XB had the same mobility as 5α-pregnane-3α,15α,20β-triol and it was eluted to give 5.2 mg of residue containing 1.05×10^5 dpm. It was rechromatographed on paper in system L for 30 hours and one symmetrical zone of radioactivity was obtained having the same mobility as 5α-pregnane-3α,15α,20β-triol. The eluate from the paper (9.8×10^4 dpm) was chromatographed on a small silica gel column and elution with 12% ethanol in methylene chloride gave 1.0 mg of colorless oil containing 9.20×10^4 dpm. An aliquot of this material (4.60×10^4 dpm) was acetylated with ^{3}H-acetic anhy-

TABLE 50

Proof of Radiochemical Purity of 3α,15α-Dihydroxy-5β-pregnan-20-one Isolated from the Glucosiduronate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mgx10^3) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3α,15α-Diacetoxy-5β-pregnan-20-one*				
1	10.9	15.2	56.6	82.0
2	8.1	10.9	27.0	35.0
3	5.6	7.5	9.7	13.5
4	5.4	7.4	5.6	7.6
3α,15α-Diacetoxy-5β-pregnan-20β-ol				
1	5.6	7.4	5.5	7.7
2	5.6	7.5	5.6	7.4
Calculated**	5.6			

*An aliquot of the purified residue in pool VI containing $4.50x10^4$ dpm was acetylated with ^3H-acetic anhydride, Batch No. 4, mixed with 50.1 mg of carrier 3α,15α-diacetoxy-5β-pregnan-20-one and the diacetate chromatographed on alumina prior to crystallization.

**The fourth crystals and mother liquor of the diacetate were combined (24.0 mg) and the mixture reduced with $NaBH_4$. The product was chromatographed on a small alumina column from which 20.0 mg of material was eluted containing $1.12x10^5$ dpm of ^3H and $1.49x10^4$ dpm of ^{14}C.

dride Batch No. 4, and the product mixed with 35.0 mg of carrier 5α-pregnane-3α,15α,20β-triacetate. The mixture was chromatographed on a small alumina column and the material eluted with benzene-Skellysolve B (2:3) was crystallized to constant specific activity as shown in Table 51. From the results shown in

TABLE 51

Proof of Radiochemical Purity of 5α-Pregnane-3α,15α,20β-triol Isolated from the Glucosiduronate Fraction of Pregnancy Urine

	Specific Activity (dpm ^3H/mgx10^3)			
Crystallization	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
5α-Pregnane-3α,15α,20β-triacetate*				
1	25.4	20.3	124.0	105.0
2	17.7	14.5	51.2	41.0
3	16.3	12.8	25.0	20.5
4	15.5	12.5	16.4	13.0

*An aliquot of XB containing $4.60x10^4$ dpm was acetylated with ^3H-acetic anhydride, Batch No. 4, and the product mixed with 35.0 mg of carrier 5α-pregnane-3α,15α,20β-triacetate. This mixture was then chromatographed on a small alumina column prior to crystallization.

this table the structure 5α-pregnane-3α,15α,20β-triol was assigned to the metabolite in zone XB. The specific activity of this metabolite was calculated to be $8.68x10^3$ dpm/μg. The material in zone XA was not identified.

Experiment C-4. Metabolism of 15α-Hydroxyprogesterone by the Normal Female

A normal 24-year old subject in the luteal phase of the menstrual cycle was injected intravenously with 3.72×10^6 dpm of (4-^{14}C)-15α-hydroxyprogesterone contained in 10.2 μg. Urine was collected for five days and the urinary conjugates hydrolyzed to give a sulfate and a glucosiduronate fraction as previously described. Table 52 shows the radioactivity in the crude urine and the sulfate and glucosiduronate extracts of each day. Since most of the radioactivity was present in the first four days' urine only the extracts from this period were further processed.

The combined sulfate fraction (248 mg, 1.78×10^5 dpm) was chromatographed on a 50 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described and the plot of radioactivity eluted versus fraction number is shown in Figure 18. The mode of elution as well as the weight and radioactivity in each pool are shown in Table 53. Because of the small amounts of radioactivity present in these pools and the unavailability of carrier steroids, only the residue obtained from pool IV was identified in the sulfate fraction.

The residue of pool IV (18.0 mg and 3.35×10^4 dpm), Figure 18, Table 53, was chromatographed on one Whatman No. 3MM paper in system A for 15 hours to give a single radioactive zone corresponding in mobility to 3β,15α-dihydroxy-5β-pregnan-20-one (3.2 mg and 2.50×10^4 dpm) and it was eluted and chromatographed on a

TABLE 52

Radioactivity in the Urine and Conjugates Excreted as Sulfates and Glucosiduronates After the Injection of (4-^{14}C)-15α-Hydroxyprogesterone to a Normal Female

Day	Crude Urine (dpm)	% of Injected Dose	Sulfates	% of Injected Dose	Glucosiduronates	% of Injected Dose
1	1.42×10^6	38.4	1.26×10^5	3.4	1.20×10^6	32.2
2	7.00×10^5	18.8	3.17×10^4	0.9	6.18×10^5	16.6
3	2.70×10^5	7.3	1.23×10^4	0.3	2.36×10^5	6.4
4	9.20×10^4	2.5	7.00×10^3	0.2	8.02×10^4	2.2
5	1.00×10^4	0.3	-	-	3.05×10^3	0.1
Total	2.49×10^6	67.3	1.78×10^5	4.8	2.14×10^6	57.6

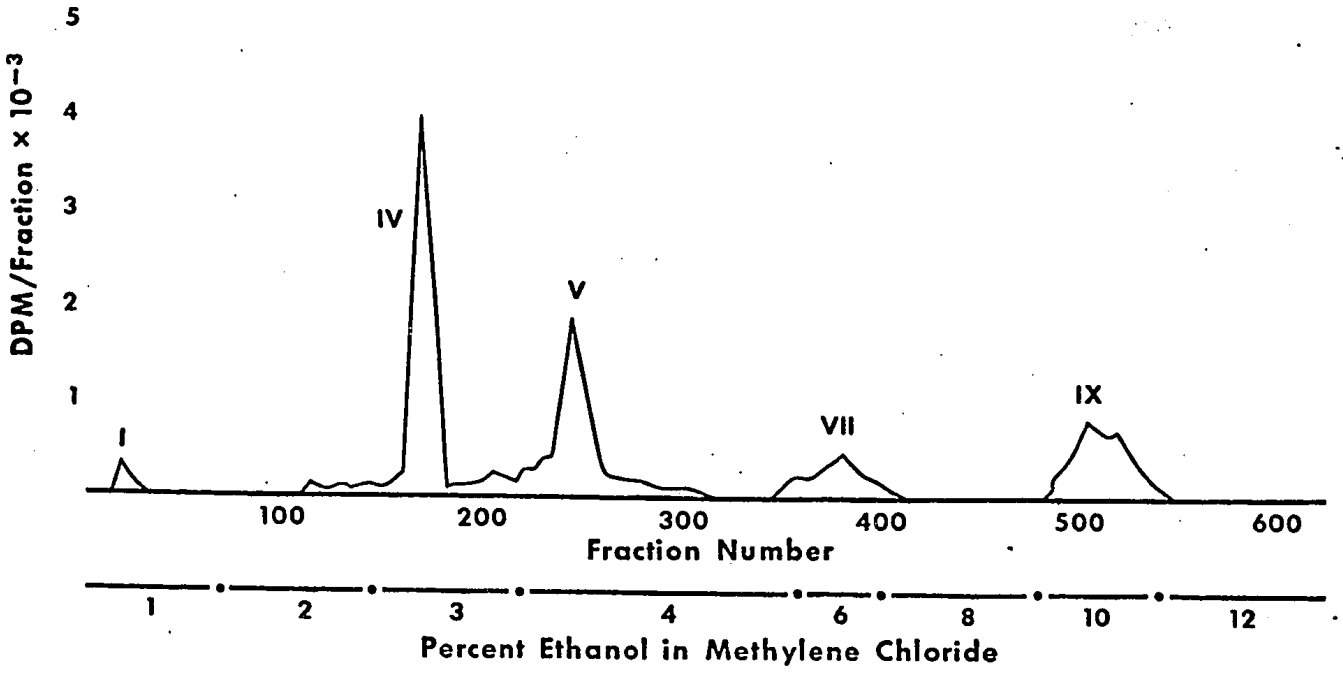


FIG. 18. Silica gel column chromatography of the sulfate fraction of urine following the administration of (4-^{14}C)-15α-hydroxyprogesterone to a non-pregnant female.

TABLE 53

Elution of Radioactive Material after Chromatography of the Sulfate Fraction of Normal Female Urine

Pool	Fraction No.	Weight	Radioactivity (dpm)
I	13-32	60.0	3.70×10^3
II	33-108	67.0	5.30×10^3
III	109-160	8.0	5.30×10^3
IV	161-182	18.0	3.35×10^4
V	183-312	25.0	3.73×104
VI	313-345	11.5	3.45×10^3
VII	346-412	23.2	1.05×10^4
VIII	413-482	16.5	4.00×10^3
IX	483-548	12.0	1.55×10^4

small silica gel column to yield 0.8 mg of colorless oil containing 1.87×10^4 dpm. This material was acetylated with ^{3}H-acetic anhydride, Batch No. 5, and the product mixed with 28.0 mg of carrier 3β,15α-diacetoxy-5β-pregnan-20-one. The mixture was chromatographed on a 3 g alumina column and the material eluted with benzene-Skellysolve B (2:1) was crystallized. Constant specific activity was achieved after the fourth crystallization and again after $NaBH_4$ reduction as shown in Table 54. From the data shown in this table, the structure 3β,15α-dihydroxy-5β-pregnan-20-one could be assigned to the metabolite in pool IV. The specific activity of this metabolite was calculated to be 3.06×10^5 dpm/μg.

TABLE 54

Proof of Radiochemical Purity of 3β,15α-Dihydroxy-5β-pregnan-20-one Isolated from the Sulfate Fraction of Normal Female Urine

		Specific Activity (dpm ^3H/mg)		
Crystallization	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3β,15α-Diacetoxy-5β-pregnan-20-one*				
1	6200	12.1	47000	92.0
2	1760	3.5	13500	27.8
3	1400	2.8	4800	9.5
4	1380	2.7	1450	2.9
3β,15α-Diacetoxy-5β-pregnan-20β-ol				
1	1370	2.7	1430	2.9
2	1410	2.7	1400	2.8
Calculated**				

*The residue from pool IV containing 1.87×10^4 dpm was acetylated with 3H-acetic anhydride, Batch No. 5, mixed with 28.0 mg of carrier 3β,15α-diacetoxy-5β-pregnan-20-one and the diacetate purified on a small alumina column prior to crystallization.

**The fourth crystals and mother liquor were combined (10.5 mg) and the mixture reduced with $NaBH_4$. The product was chromatographed on a small alumina column from which 8.0 mg of material was eluted containing 1.12×10^4 dpm of ^3H and 4.00×10^3 dpm of ^{14}C.

The combined glucosiduronate fraction (339 mg) was chromatographed on a 60 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. In Figure 19 is shown the pattern of elution of radioactivity from the column. The mode of elution, the weight and radioactivity of each pool are shown in Table 55. Pools I, V and VI contained

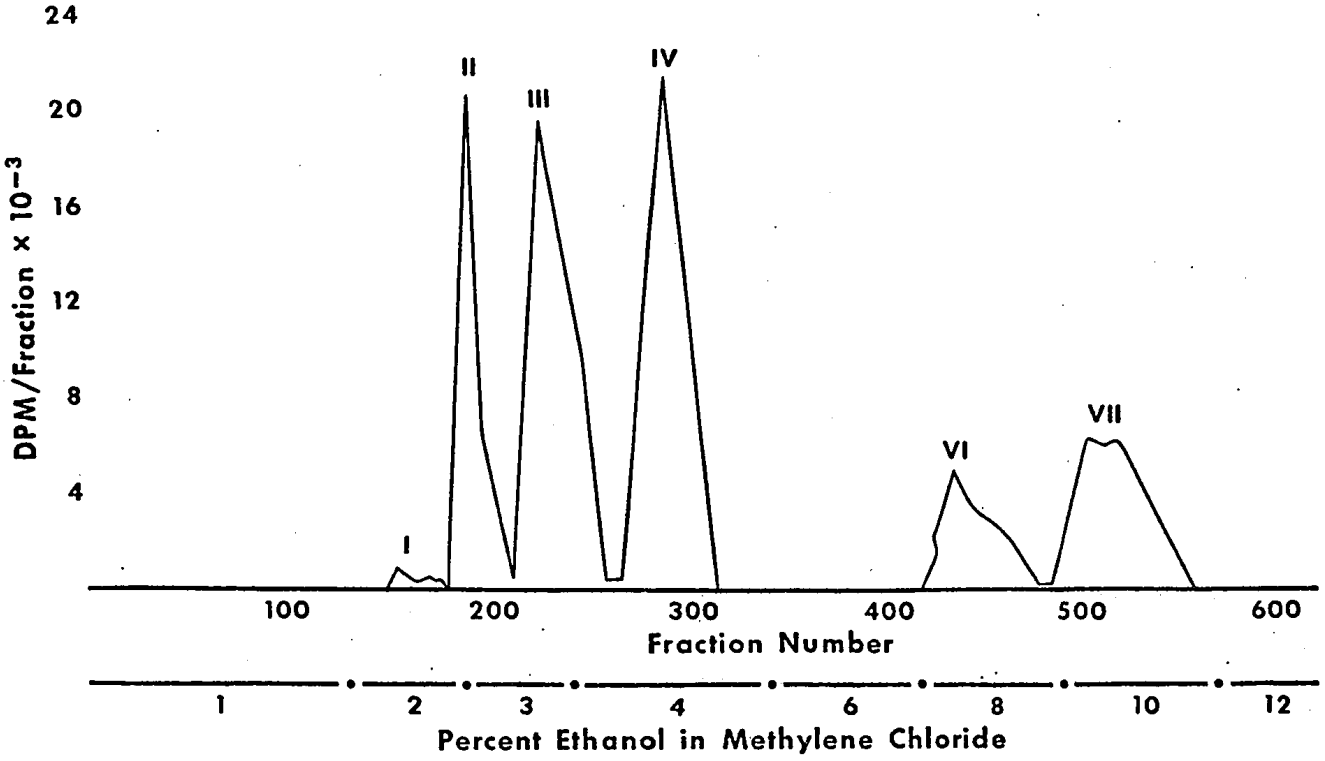


FIG. 19. Silica gel column chromatography of the glucosiduronate fraction of urine following the administration of (4-^{14}C)-15α-hydroxyprogesterone to a non-pregnant female.

TABLE 55

Elution of Radioactive Material After Silica Gel Column Chromatography of the Glucosiduronate Fraction of Normal Female Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	151-180	53.5	3.22×10^4
II	181-215	28.0	3.35×10^5
III	216-265	33.0	5.07×10^5
IV	266-316	25.5	4.82×10^5
V	317-422	62.3	6.32×10^4
VI	423-485	15.4	1.17×10^5
VII	486-560	11.2	2.83×10^5

radioactive material which could not be identified.

The residue of pool II (28.0 mg and 3.35×10^5 dpm) was chromatographed on two Whatman No. 3MM papers in system A for 10 hours to yield one main radioactive zone corresponding in mobility to 15α-hydroxyprogesterone. The material eluted from the papers weighed 9.2 mg and contained 2.75×10^5 dpm. It was chromatographed on a single paper in system B for 5 days and one peak of radioactivity was observed having the same mobility as 15α-hydroxyprogesterone. The material eluted (2.8 mg and 2.38×10^5 dpm) was chromatographed on a small alumina column and elution with 1.5% ethanol in benzene yielded 0.8 mg of residue containing 2.10×10^5 dpm. An aliquot of this residue containing 7.00×10^4 dpm was acetylated with ^{3}H-acetic anhydride,

Batch No. 5, and the product mixed with 50.5 mg of carrier 15α-acetoxyprogesterone. The mixture was chromatographed on a 6 g alumina column and the material eluted with benzene-Skellysolve B (4:1) was crystallized. Constant specific activity was achieved after the fourth crystallization and again after $NaBH_4$ reduction and DDQ oxidation of the acetate as shown in Table 56. The specific activity of the urinary 15α-hydroxyprogesterone was calculated to be 3.31×10^5 dpm/μg.

TABLE 56

Proof of Radiochemical Purity of 15α-Hydroxyprogesterone Isolated From the Glucosiduronate Fraction of Normal Female Urine

	Specific Activity (dpm ^3H/mg)			
Crystallization	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
15α-Acetoxyprogesterone*				
1	14100	10.0	34200	25.0
2	3020	2.2	25500	18.5
3	1900	1.3	7200	5.1
4	1850	1.3	1930	1.4
15α-Acetoxy-20β-hydroxypregn-4-en-3-one				
1	1510	1.2	1680	1.4
2	1490	1.2	1540	1.3
Calculated**	1550			

*An aliquot of II containing 7.00×10^4 dpm was acetylated with ^3H-acetic anhydride, Batch No. 5, and the product was mixed with 50.0 mg of carrier 15α-acetoxyprogesterone. The mixture was chromatographed on a small alumina column prior to crystallization.

**The fourth crystals and mother liquor were combined (21.0 mg) and the mixture reduced with $NaBH_4$ and then oxidized with the DDQ reagent. The product was chromatographed on a small alumina column to yield 14.5 mg of oily material containing 2.25×10^4 dpm of ^3H and 1.88×10^4 dpm of ^{14}C.

Chromatography of the residue of pool III, Figure 19, Table 55, on two Whatman No. 3MM papers in system A for 24 hours afforded one radioactive zone corresponding in mobility to 3α,15α-dihydroxy-5α-pregnan-20-one. The material eluted (11.5 mg and 4.25×10^5 dpm) was chromatographed on a single paper in system L for 8 hours and again one major peak of radioactivity was observed having the same mobility as 3α,15α-dihydroxy-5α-pregnan-20-one. The eluate (4.5 mg and 3.72×10^5 dpm) was chromatographed on a small silica gel column and elution with 3% ethanol in methylene chloride afforded 2.5 mg of yellow oil containing 3.25×10^5 dpm. An aliquot of this material containing 6.50x1 dpm was acetylated with ^{3}H-acetic anhydride, Batch No. 5, and the product was mixed with 31.0 mg of carrier 3α,15α-diacetoxy-5α-pregnan-20-one. After chromatography on a small alumina column the diacetate eluted with benzene-Skellysolve B (1:1) was crystallized to constant specific activity and the process of crystallization was repeated after $NaBH_4$ reduction as shown in Table 57. From the data shown in this table it was possible to assign the structure of 3α,15α-dihydroxy-5α-pregnan-20-one to the metabolite in pool III. The specific activity of this metabolite was calculated to be 3.45×10^5 dpm/μg.

The residue of pool IV, Figure 19, Table 55, was chromatographed on three papers in system A for 24 hours and one major radioactive zone having the same mobility as 3α,15α-dihydroxy-5β-pregnan-20-one was obtained. The material eluted (5.2 mg and 3.82 $\times 10^5$ dpm) was chromatographed on a small silica gel column and elution with 4% ethanol in methylene chloride afforded 2.8 mg of

TABLE 57

Proof of Radiochemical Purity of 3α,15α-Dihydroxy-5α-pregnan-20-one Isolated from the Glucosiduronate Fraction of Normal Female Urine

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3α,15α-Diacetoxy-5α-pregnan-20-one*				
1	19400	11.2	68400	38.0
2	7700	4.5	45500	26.0
3	4730	2.8	20900	12.3
4	4340	2.6	4670	2.7
3α,15α-Diacetoxy-5α-pregnan-20β-ol				
1	4060	2.4	4880	2.8
2	4090	2.4	4190	2.4
Calculated**	4200			

*An aliquot of III containing 6.50×10^4 dpm was acetylated with ^3H-acetic anhydride, Batch No. 5, mixed with 31.0 mg of carrier 3α,15α-diacetoxy-5α-pregnan-20-one, and the mixture chromatographed on an alumina column prior to crystallization

**The fourth crystals and mother liquor were combined (11.5 mg) and the mixture reduced with $NaBH_4$. The product was then chromatographed on a small alumina column from which 9.3 mg of material was eluted containing 3.91×10^4 dpm of ^3H and 1.63×10^4 dpm of ^{14}C.

residue containing 3.52×10^5 dpm. An aliquot of this residue containing 7.04×10^4 dpm was acetylated with ^3H-acetic anhydride, Batch No. 5, and the product was mixed with 50.2 mg of carrier 3α,15α-diacetoxy-5β-pregnan-20-one. The mixture was chromatographed on a 6 g alumina column and the material eluted with benzene-Skellysolve B (1:1) was crystallized. Constant specific

activity was achieved after the fourth crystallization and again after $NaBH_4$ reduction of the diacetate as shown in Table 58.

TABLE 58

Proof of Radiochemical Purity of 3α,15α-Dihydroxy-5β-pregnan-20-one Isolated from the Glucosiduronate Fraction of Normal Female Urine

Crystallization	Specific Activity (dpm ^3H/mg) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3α,15α-Diacetoxy-5β-pregnan-20-one*				
1	10400	9.5	52500	43.0
2	3460	3.2	23500	21.0
3	2810	2.6	11800	10.3
4	2780	2.5	3000	2.6
3α,15α-Diacetoxy-5β-pregnan-20β-ol				
1	2530	2.3	2880	2.5
2	2560	2.4	2690	2.4
Calculated**	2700			

*An aliquot of IV containing 7.04×10^4 dpm was acetylated with ^3H-acetic anhydride, Batch No. 5. The product was mixed with 50.2 mg of carrier 3α,15α-diacetoxy-5β-pregnan-20-one and the mixture was chromatographed on a small alumina column prior to crystallization.

**The fourth crystals and mother liquor were combined (21.5 mg) and the mixture reduced with $NaBH_4$. The product was chromatographed on a small alumina column from which 16.3 mg of material was eluted containing 4.20×10^4 dpm of ^3H and 1.76×10^4 dpm of ^{14}C.

From the data shown in this table it was possible to assign the structure 3α,15α-dihydroxy-5β-pregnan-20-one to the metabolite in pool IV. The specific activity of this metabolite was calcu-

lated to be 3.52×10^6 dpm/μg.

Further purification of the residue of pool VII, Figure 19, Table 55, was accomplished by chromatography on one paper in system A for 8 days. Two major zones of radioactivity, VIIA and VIIB, were obtained at average distances of 8.3 and 22.0 cm, respectively. Zone VIIB corresponded in mobility to 5α-pregnane-3α,15α,20β-triol and it was eluted to give a residue which weighed 5.1 mg and contained 1.55×10^5 dpm. Chromatography of this residue on paper in system L for 30 hours afforded one radioactive zone with the mobility of 5α-pregnane-3α,15α,20β-triol. The material eluted (3.7 mg and 1.40×10^5 dpm) was further purified by chromatography on a small silica gel column. Elution with 10% ethanol in methylene chloride gave 1.8 mg of an oily residue containing 1.22×10^5 dpm. An aliquot of this residue containing 4.08×10^4 dpm was acetylated with ^{3}H-acetic anhydride, Batch No. 5, and the product was mixed with 41.2 mg of carrier triacetate. The mixture was chromatographed on a small alumina column and the material eluted with benzene-Skellysolve B (2:3) was crystallized to constant specific activity as shown in Table 59.

From the data shown in this table, the structure 5α-pregnane-3α,15α,20β-triol could be assigned to the metabolite in zone VIIB. The specific activity of this metabolite was calculated to be 3.35×10^5 dpm/μg. The material in zone VIIA was not identified.

TABLE 59

Proof of Radiochemical Purity of 5α-Pregnane-3α,15α,20β-triol Isolated from the Glucosiduronate Fraction of Normal Female Urine

Crystallization	Specific Activity (dpm ^3H/mg) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
5α-Pregnane-3α,15α,20β-triacetate*				
1	9200	13.0	53000	70.5
2	4480	6.5	20400	28.5
3	2660	3.8	12800	18.0
4	2660	3.7	2650	3.8

*An aliquot of VIIB containing 4.08×10^4 dpm was acetylated with ^3H-acetic anhydride, Batch No. 5. The product was mixed with 41.2 mg of carrier 5α-pregnane-3α,15α,20β-triacetate and the mixture chromatographed on a small alumina column prior to crystallization.

DISCUSSION

In planning these studies it was immediately evident that a facile method had to be developed for the preparation of labeled 15α-hydroxysteroids to be used as precursors. The ability of certain micro-organisms to introduce hydroxyl groups into the steroid molecule is well known (2). Hence, Penicillium (ATCC 11598), generously provided by Dr. P.A. Diassi, was used to effect the conversion of labeled precursors to the 15α-hydroxylated products. Labeled 15α-hydroxyprogesterone and 15α-hydroxyandrostenedione were thus prepared in good yield and with a high degree of purity. The same method was also used to prepare large amounts of non-labeled 15α-hydroxyprogesterone. When these studies were initiated, the first batch of (7-^{3}H)-15α-hydroxyprogesterone was prepared for us through the courtesy of Dr. P.A. Diassi who used the micro-organism Colletotrichum linicola. Thereafter the labeled 15α-hydroxysteroids were prepared in our own laboratory. The specific activities of the labeled 15α-hydroxysteroids were accurately determined by acetylation of an aliquot of the purified steroid with acetic anhydride of known specific activity containing a different label, followed by addition of the appropriate carrier acetate and crystallization to constant ^{3}H/^{14}C ratio. The crystallization procedure was then repeated after the formation of a derivative.

Not many urinary steroids retaining the Δ^{4}-3-ketone group have been isolated. Steroids such as cortisol (47) and aldos-

sterone (48) are excreted into the urine in small quantities. There are two steroids, 6β-hydroxycortisol (49) and 16α-hydroxyprogesterone (40) which are present in the urine in somewhat higher concentrations.

The difficulty in the isolation of a steroid present in the urine in very small amounts is due to bulky extracts which have to be processed because large volumes of urine have to be used to obtain the quantities sufficient for chemical identification. This difficulty was in part circumvented in these studies, because very small amounts of 15α-hydroxyprogesterone, 15α-hydroxyandrostenedione and 15α-hydroxytestosterone were detected in relatively small pools of urine by the use of the isotope dilution procedure (43). In this procedure small amounts of the labeled steroid of known specific activity were added to the urine prior to the hydrolysis of the urinary steroid conjugates and the extraction of the resulting unconjugated steroids with ethyl acetate. The neutral extract thus obtained was chromatographed first on a large silica gel column on which the fraction containing the radioactive material was separated from the bulk of urinary steroids and this fraction was further purified by paper chromatography. The purified extract containing the radioactive material was acetylated with acetic anhydride of known specific activity containing a second label (^{3}H or ^{14}C). After the addition of carrier acetate, the mixture was crystallized to constant specific activity and constant $^{3}H/^{14}C$ ratios and the crystallization procedure was repeated following the formation of an appropriate derivative. From the

final $^{3}H/^{14}C$ ratio and the amount of radioactivity originally added to the urine it was possible to calculate the amount of the steroid excreted in the urine.

This method facilitated the detection of small amounts of steroids in the urine since in every chromatographic step the radioactivity was easily followed by counting of fractions from column chromatograms or by scanning paper. Moreover, since the labeled steroid added to the urine was mixed with the excreted urinary steroid, losses occurring during purification could be corrected and quantitative results were obtained. However, possible losses due to the degree and specificity of the hydrolytic procedure used are not corrected for by this method. In initial experiments, Glusulase (a mixture of β-glucuronidase and sulfatase) was used for the hydrolysis of the urinary steroid conjugates. Following the injection of (4-^{14}C)-15α-hydroxyprogesterone to a pregnant female, 15α-hydroxyprogesterone was found only in the glucosiduronate fraction and not in the sulfate fraction of the urine. Hence, in some isolation studies only β-glucuronidase was used for the hydrolysis of the urinary steroid conjugates. However, other conjugated forms of urinary 15α-hydroxyprogesterone cannot be excluded. Optimal quantitation can only be achieved if the labeled steroid is added to the urine in the form of the conjugate(s) excreted.

The addition to the urine of a known amount of the labeled steroid is a critical step in this quantitative procedure since an error in this figure would lead to inaccuracies in the final calculation. In every experiment, an aliquot of the radioactive material was added to the urine and at the same time two to four

aliquots of the material were taken for counting. In all cases, the aliquots counted did not differ more than 2%. Thus, the error of pipetting the radioactive material to the urine was always less than 2%. It should also be mentioned that following hydrolysis the urinary steroids were extracted with ethyl acetate which was then washed with 0.5N NaOH to remove pigments and phenolic steroids. The 15α-hydroxylated steroids were stable to alkali of this normality because no breakdown products were observed when labeled 15α-hydroxylated steroids were added to the urine and the neutral extract was prepared in this manner.

In the first study discussed, 110 μg of crystalline 15α-hydroxy progesterone was isolated from a 14-day urine pool obtained from a subject in the third trimester of pregnancy and it was identified by its mp, mmp and infrared spectrum. In all the other isolation studies the isotope dilution technique was used to quantitate the amount of this steroid excreted in the urine.

Having isolated crystalline 15α-hydroxyprogesterone from late pregnancy urine, we then proceeded to quantitate the amount of this steroid excreted in a large number of urine pools obtained from subjects with a variety of endocrine status. The urines from the following subjects were analyzed: Normal females in the follicular and luteal phases of the menstrual cycle, females in the first, second and third trimester of pregnancy, patients with Cushing's syndrome, one patient with con-

genital adrenal hyperplasia having a C-21 block but no salt loss, and newborn infants. The excretion of 15α-hydroxyprogesterone in the urine of these subjects is shown in Table 60.

TABLE 60

Excretion of Urinary 15α-Hydroxyprogesterone

	µg/day
Pregnancy	
3rd trimester (2)*	28-34
2nd trimester (2)	5-6
1st trimester (2)	0-3
Normal female, follicular phase (2)	0
Normal female, luteal phase (2)	0
Cushing's Syndrome (2)	1-2
Congenital Adrenal Hyperplasia (1)	0
Newborn infants (1)	0.6

*Numbers in parentheses denote the number of determinations performed.

The striking feature of the results shown in this table is that 15α-hydroxyprogesterone was isolated in significant amounts only from the urine of subjects in the second and third trimester of pregnancy. Small amounts of 15α-hydroxyprogesterone (5-6 µg/day) were isolated from the urine of subjects in the second trimester of pregnancy and the largest amounts (28-34 µg/day) were isolated from late pregnancy urine. Insignificant amounts of 15α-hydroxyprogesterone were isolated from the urine of

normal females, subjects with Cushing's syndrome, the subject with congenital adrenal hyperplasia and newborn infants. From the urine of one subject in the 13th week of gestation very small amounts (3 μg/day) of 15α-hydroxyprogesterone were isolated, but this steroid could not be detected in the urine of a subject in the 8th week of gestation. These results demonstrate that 15α-hydroxyprogesterone is present in the urine after the 8th week of pregnancy and reaches maximal values in late pregnancy. These results are consistent with the published data for the isolation of 15α-hydroxylated estrogens which are excreted in late pregnancy urine but were not found in the urine of normal females and of pregnant females in the second trimester of pregnancy (20). Since very little, if any, 15α-hydroxyprogesterone was detected in the urine of newborn infants, the placenta as well as fetal tissues may be involved in the formation of this steroid. It is worthwhile to mention that although 15α-hydroxyprogesterone was not found in the urine of the patient with congenital adrenal hyperplasia, relatively large amounts of 16α-hydroxyprogesterone were isolated in this study. This is the first time that 16α-hydroxyprogesterone has been isolated from the urine of such subjects.

Following the perfusion of mid-term previable human fetuses with labeled testosterone and androstenedione, Mancuso et al (25) isolated 15α-hydroxyestradiol from the fetal liver, but 15α-hydroxyandrostenedione or 15α-hydroxytestosterone could not be detected. When the mid-term previable human fetus was per-

fused with ^{14}C-progesterone (17), 15α-hydroxyprogesterone could not be found in all of the fetal tissues examined. The finding that 15α-hydroxyprogesterone is present in late pregnancy urine prompted the next studies which were to isolate 15α-hydroxyandrostenedione and 15α-hydroxytestosterone from this source. In two such studies we isolated very small amounts (4-5 μg/day) of 15α-hydroxyandrostenedione and much larger amounts (30 μg/day) of 15α-hydroxytestosterone. The presence of these steroids in late pregnancy urine raise the possibility that they may be important precursors of the 15α-hydroxylated estrogens found in the urine of pregnant women during the late stages of gestation. This was borne out by the findings of Stern, Givner and Solomon (26) when they showed that 15α-hydroxyandrostenedione incubated with the 10,000 x g supernatant fraction of human placental tissue was converted mainly to 15α-hydroxyestradiol (30-40%) and to a much smaller extent (0.2-0.3%) to 15α-hydroxytestosterone. No 15α-hydroxyestrone was isolated in these studies. This latter finding may explain why only very small amounts of 15α-hydroxyandrostenedione and much larger amounts of 15α-hydroxytestosterone were found in late pregnancy urine and suggests that 17β-hydroxysteroid dehydrogenase activity present in the placenta may convert 15α-hydroxyandrostenedione to 15α-hydroxytestosterone. Further evidence supporting the possibility that neutral steroids may serve as important precursors of the 15α-hydroxylated estrogens was provided by Gurpide et al (11) who showed that dehydroiso-

androsterone sulfate was converted (to a small extent) to 15α-hydroxyestriol and the results of YoungLai and Solomon (27) who isolated radioactive 15α-hydroxyestriol from the urine following the intravenous injection of labeled dehydroisoandrosterone sulfate and 16α-hydroxydehydroisoandrosterone sulfate to a female in the third trimester of pregnancy.

The isolation of 15α-hydroxyprogesterone from late pregnancy urine and the failure to detect it in the urine of non-pregnant subjects suggest that this steroid is elaborated in the fetoplacental unit. In view of the large amounts of progesterone produced during pregnancy, it seemed reasonable to suppose that circulating progesterone could serve as a precursor of urinary 15α-hydroxyprogesterone. In order to obtain information as to whether this conversion occurs in the fetoplacental unit or in the mother, or both, an experimental design was employed which was similar to that used by Gurpide et al (11) for their study on the origin of 15α-hydroxyestriol. With the collaboration of Dr. J. Bowman, Rh Laboratory, Winnipeg, Manitoba, three such studies were performed. In two studies, ^{3}H-progesterone was injected into the peritoneal cavity of the fetus at the time of amniocentesis and at the same time ^{14}C-progesterone was injected into an antecubital vein of the mother. Urine was collected for five days following the injections of the labeled precursors and 15α-hydroxyprogesterone containing ^{3}H and negligible amounts of ^{14}C was isolated from the glucosiduronate fraction of the urine. The minimum con-

version of the injected ^{3}H-progesterone to urinary 15α-hydroxyprogesterone was 0.014%. This indicated that the conversion of progesterone to 15α-hydroxyprogesterone occurred in the feto-placental unit. In the third study, ^{3}H-progesterone was again administered into the peritoneal cavity of the fetus and a small amount (9,000 dpm) of ^{14}C-15α-hydroxyprogesterone was injected intravenously in the mother. Again urine was collected for five days following the injections and 15α-hydroxyprogesterone containing both ^{3}H and ^{14}C with a $^{3}H/^{14}C$ dpm ratio of 0.42 was isolated from the glucosiduronate fraction of the urine. The accuracy of counting ^{3}H and ^{14}C with low $^{3}H/^{14}C$ ratio was checked by counting standards separately and in mixtures with a dpm ratio of approximately 0.4. Under these conditions the accuracy of counting ^{3}H and ^{14}C was about 5%. Using the final $^{3}H/^{14}C$ ratio of the isolated 15α-hydroxyprogesterone and the injected doses of ^{3}H-progesterone and (4-^{14}C)-15α-hydroxyprogesterone, it was calculated that the conversion of ^{3}H-progesterone to 15α-hydroxyprogesterone was 0.076%.

These results are similar to those obtained by Gurpide et al (11) who injected ^{3}H-estradiol into the fetus and ^{14}C-estradiol in the mother and found that the isolated 15α-hydroxyestriol contained only ^{3}H. These authors concluded that 15α-hydroxyestriol is elaborated in the fetus. Our studies also indicate that 15α-hydroxyprogesterone is of fetoplacental origin and that very little, if any, 15α-hydroxyprogesterone is made by the maternal tissues. Schneider (22) regarded 15α- and 15β-

hydroxylations as primitive reactions in an evolutionary sense because he could not demonstrate such hydroxylations in mammalian tissues but showed that deoxycorticosterone was converted to 15α- and 15β-hydroxydeoxycorticosterone after incubation of the substrate with liver slices of the American bullfrog. However in recent years it has been demonstrated that the adult human adrenal (7) and the human fetal liver (8) can 15α-hydroxylate estrogens and a number of 15α- and 15β-hydroxylated estrogens has been isolated from human late pregnancy urine (9-15). Moreover, in the studies reported in this thesis, 15α-hydroxyprogesterone, 15α-hydroxyandrostenedione and 15α-hydroxytestosterone were isolated from human late pregnancy urine and it was also demonstrated that progesterone was converted to 15α-hydroxyprogesterone in the human fetoplacental unit. From these findings it is clear that 15-hydroxylation of steroids is not confined to the lower species.

Having determined that 15α-hydroxyprogesterone was present in late pregnancy urine and formed in the fetoplacental unit, we then turned our attention to the <u>in vivo</u> metabolism of this interesting steroid. Since 15α-hydroxylated neutral steroids could not be obtained commercially, or from other investigators, the first two experiments were designed to isolate sufficient weight in the urinary metabolites of 15α-hydroxyprogesterone for the purpose of their identification. In these two experiments, the urinary steroid conjugates were hydrolyzed with Glusulase. Except for a few modifications, the methods used for the isolation of the urinary metabolites were the same as those described

by Ruse and Solomon (50) and they proved to be quite effective in these experiments. Since no standards were available for comparison, the structures of the metabolites were established by well accepted methods employing various physical methods of analysis such as infrared spectroscopy, mp determinations, nuclear magnetic resonance spectroscopy, mass spectroscopy and optical rotation studies. A combination of these physical procedures provided unequivocal proof of the identity of the urinary metabolites as the free steroids and as the acetates.

Generally, the following procedure was employed in the identification of the metabolites of 15α-hydroxyprogesterone : The metabolite was isolated in a crystalline form and was subjected to infrared analysis. The infrared spectrum afforded information with regard to the presence of hydroxyl groups, ketonic groups and the retention or reduction of the Δ^4-3-keto group. An aliquot of the metabolite was acetylated with labeled acetic anhydride and the specific activity of the acetate was determined. From this specific activity it was possible to calculate the number of acylable hydroxyl groups. Infrared spectroscopy studies on the acetate gave information whether the metabolite contained non-acylable (hindered) hydroxyl groups. None of the metabolites isolated in these studies had hindered hydroxyl groups. At this stage aliquots of the free and the acetylated metabolite were sent to Dr. L. Durham for nuclear magnetic resonance and mass spectroscopy studies from which final identification was achieved.

When the metabolite contained a 20-hydroxyl group, the

information obtained from nuclear magnetic resonance studies was not conclusive for the assignment of the configuration of the hydroxyl group at the C20 position. Two such metabolites were isolated, namely, 5α-pregnane-3α,15α,20-triol and 5β-pregnane—3α,15α,20-triol. When an aliquot of 3α,15α-diacetoxy-5α-pregnan-20-one was reduced with $NaBH_4$ and the product acetylated, the resulting material had an infrared spectrum almost identical to that of 5α-pregnane-3α,15α,20-triacetate (see Figure 11, page 117). It is very possible that the small differences observed in the infrared spectra of these two compounds are due to the presence of impurities since only small amounts of the triol were isolated and the material could only be crystallized once. Since it is known that $NaBH_4$ reduction of a 20-ketone favours formation of the 20β-hydroxyl, the information obtained above suggested that the isolated metabolite was a 20β-alcohol. To study the effect of the 15α-hydroxyl group on the $NaBH_4$ reduction of the 20-ketone, an aliquot of 15α-acetoxyprogesterone was reduced with $NaBH_4$ followed by oxidation with DDQ and a second aliquot was incubated with 20β-hydroxysteroid dehydrogenase. The products obtained by reduction with $NaBH_4$ followed by DDQ oxidation and by the enzymatic reduction had identical infrared spectra and the same mp and mixed mp on admixture. It was therefore concluded that $NaBH_4$ reduction of a 15α-hydroxy-20-ketosteroid results in the formation of the 20β-alcohol. Further information on the configuration of the 20-hydroxyl in the isolated triol was obtained by optical rotation studies. Fieser and Fieser (46) summarized the MD data for nineteen pairs of 20-epimers in the pregnane series and pointed out that the shift

in M_D on acetylation is positive for 20β-ols and negative for 20α-ols. Table 40, page 122, shows the shift in M_D on acetylation of 20α-dihydroprogesterone and 20β-dihydroprogesterone. The same correlation reported by Fieser and Fieser was observed in that the ΔM_D for the 20α-epimer was slightly negative (-4^o) while the ΔM_D for the 20β-epimer was positive ($+212^o$). When an aliquot of 15α-acetoxyprogesterone was reduced with $NaBH_4$ the resulting 20-alcohol and its acetate had a ΔM_D of +179 as shown in Table 40. These observations provide further supporting evidence that $NaBH_4$ reduction of 15α-hydroxy-20-keto-steroids results in the formation of the 20β-alcohols. Thus the structure 5α-pregnane-3α,15α,20β-triol was assigned to the isolated metabolite.

The second metabolite isolated, which had the C_{20} ketone reduced, was 5β-pregnane-3α,15α,20-triol. Again the information obtained from nuclear magnetic resonance studies did not permit the assignment of the configuration of the 20-hydroxyl group. When an aliquot of the isolated 3α,15α-dihydroxy-5β-pregnan-20-one was reduced with $NaBH_4$, the product obtained after reduction had an infrared spectrum (KBr) almost identical to that of the isolated triol (see Figure 14, page 134). The infrared spectra (CS_2) of the triacetate of the isolated triol and the triacetate formed after $NaBH_4$ reduction of 3α,15α-dihydroxy-5β-pregnan-20-one were again almost identical (see Figure 15, page 135). It is very possible that the small differences observed in the infrared spectra of these two compounds are due to the presence of impurities since only small amounts of the triol were isolated

and the material could only be crystallized once. Since it was established that $NaBH_4$ reduction of 15α-hydroxy-20-keto-steroids results in the formation of 20β-alcohols, the structure 5β-pregnane-3α,15α,20β-triol was assigned to the isolated metabolite.

Following the oral ingestion of 800 mg of 15α-hydroxy-progesterone and the simultaneous intravenous injection of the labeled steroid to a normal male, it was possible to isolate and identify four metabolites which are listed in Table 61. These are 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3α,15α,20β-triol. This table shows the specific activities of the isolated metabolites and the amounts isolated expressed as a percentage of the injected radioactivity. All of these metabolites have not hitherto been described as urinary steroids. Although the specific activities of the various metabolites were different, they were all of the same order of magnitude. However no meaningful explanation can be offerred for these differences because of the large load of orally administered steroid and because of the possibility that absorption across the intestine was not uniform with time.

The second experiment in this series was designed to confirm the results obtained in the first study, and also to isolate larger amounts of the urinary metabolites for use as carriers in the identification of the urinary metabolites of (4-^{14}C)-15α-hydroxyprogesterone described in Experiments C-3 and C-4.

TABLE 61

Specific Activities of Metabolites Isolated from Normal Male Urine after the Injection of (7-^{3}H)-15α-Hydroxyprogesterone and the Oral Ingestion of 800 mg of Carrier Steroid

Metabolite	Specific Activity (dpm/mg)	% Of Injected Radioactivity*
15α-Hydroxyprogesterone	1.08×10^5	12.9
3α,15α-Dihydroxy-5α-pregnan-20-one	1.42×10^5	8.7
3α,15α-Dihydroxy-5β-pregnan-20-one	7.90×10^4	7.9
5α-Pregnane-3α,15α,20β-triol	1.96×10^5	1.4

*These values were computed from the specific activities and weights of the crystalline metabolites and the radioactivity injected.

In this study, each of four normal males were given an oral dose of about 800 mg of 15α-hydroxyprogesterone but no labeled steroid was injected intravenously. The urinary metabolites isolated in this experiment are listed in Table 62. The first four metabolites isolated in this experiment were also isolated in the first study. However, in this experiment two additional metabolites, namely 3β,15α-dihydroxy-5β-pregnan-20-one and 5β-pregnane-3α,15α,20β-triol were isolated and identified. The triol was also isolated in the first study but in too small an amount to permit its identification, while 3β,15α-dihydroxy-5β-pregnan-20-one was not isolated in the first experiment.

The two studies described above provided information on the metabolism of 15α-hydroxyprogesterone given orally to

TABLE 62

Metabolites Isolated from the Urine of Four Normal Males Following the Oral Ingestion of a Total of 3.26 g of 15α-Hydroxyprogesterone

Metabolite	Weight of Crystalline Material Isolated (mg)	mp (°C)
15α-Hydroxyprogesterone	145	230-232
3α,15α-Dihydroxy-5α-pregnan-20-one	179	175-177
3α,15α-Dihydroxy-5β-pregnan-20-one	402	208-210
5α-Pregnane-3α,15α,20β-triol	9	211-214
5β-Pregnane-3α,15α,20β-triol*	18.5	-
3β,15α-Dihydroxy-5β-pregnan-20-one	45	193-195

*This metabolite could not be crystallized. The weight reported is the weight of the purified residue.

normal males and also permitted the isolation of large amounts of these metabolites which could be used as standards for the study of the metabolism of tracer doses of (4-^{14}C)-15α-hydroxyprogesterone administered intravenously to a pregnant female and to a non-pregnant female. In these latter studies, the urinary steroid conjugates were cleaved first by solvolysis and then by β-glucuronidase hydrolysis. It is therefore possible that the glucosiduronate fraction contained some metabolites which were excreted in the urine conjugated with both sulfuric and glucosiduronic acids. It is interesting to note that in both experiments the excretion of radioactivity in the 4-day urine collection was more than 65%. Of this amount, over 50% was

present in the urine of the first day in the case of the non-pregnant female, and about 95% was present in the urine of the first day in the case of the pregnant female. These results indicate that the urinary metabolites of the injected 15α-hydroxyprogesterone were cleared rapidly. In both studies most of the radioactivity was found in the glucosiduronate fraction and a small percentage of the injected dose was found in the sulfate fraction of the urine. Since only minute amounts of the metabolites were isolated in these studies, their identification and the determination of their specific activities was accomplished by the isotope dilution procedure. The purified metabolite containing ^{14}C was acetylated with tritium labeled acetic anhydride of known specific activity and the acetylation product mixed with the appropriate carrier acetate. These acetates were prepared by acetylation of the metabolites isolated in Experiment C-2. The mixture was then crystallized to constant specific activities and constant $^{3}H/^{14}C$ ratios and the same procedure was repeated following the formation of a derivative. Using the final $^{3}H/^{14}C$ ratio, it was possible to calculate the amount of the metabolite isolated.

With the exception of 5β-pregnane-3α,15α,20β-triol, all of the metabolites isolated from the urine of the normal male given the steroid per os were present in the urine of the pregnant female who was given an intravenous injection of (4-^{14}C)-15α-hydroxyprogesterone (specific activity 3.66×10^{5} dpm/μg, contained in 13.4 μg). In this experiment, 3β,15α-dihydroxy-5β-pregnan-20-one was isolated from the sulfate fraction and

15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3α,15α,20β-triol were isolated from the glucosiduronate fraction of the urine. As shown in Table 63, the specific activities of the isolated metabolites were from 25 to 45 times lower than that of the injected 15α-hydroxyprogesterone demonstrating that all these steroids are normal constituents of human late pregnancy urine. In addition, the specific activities of all the metabolites were very similar with the exception of 15α-hydroxyprogesterone which had a higher specific activity. These results suggest that the metabolites of 15α-hydroxyprogesterone may in part be derived from precursors other than 15α-hydroxyprogesterone, such as 15α-hydroxypregnenolone. It is also possible that the lower specific activities of these metabolites may be due to differences in the metabolism of 15α-hydroxyprogesterone in the fetal and maternal compartments and different rates of transplacental passage of the metabolites from the fetal to the maternal circulation. Assuming that urinary 15α-hydroxyprogesterone is a unique metabolite of endogenous 15α-hydroxyprogesterone, the production rate of this steroid was calculated to be 79 μg/day using the well known expression for a single compartment:

$$\text{Production rate} = \frac{\text{Radioactivity Injected}}{\text{Specific activity of urinary steroid} \times \text{days of urine collection}} .$$

It is interesting to note that the major metabolites of 15α-hydroxyprogesterone are the two dihydroxy-ketones, namely, 3α,15α-dihydroxy-5α-pregnan-20-one and 3α,15α-dihydroxy-5β-

TABLE 63

Metabolites Isolated from the Urine of a Pregnant Female After Injection of (4-^{14}C)-15α-Hydroxyprogesterone*

Metabolite	Specific Activity (dpm/μg) Sulfate	Glucosiduronate	% Conversion of Injected Dose
15α-Hydroxyprogesterone	-	1.56×10^4	18.7
3α,15α-Dihydroxy-5α-pregnan-20-one	-	7.90×10^3	2.9
3α,15α-Dihydroxy-5β-pregnan-20-one	-	9.70×10^3	7.5
3β,15α-Dihydroxy-5β-pregnan-20-one	8.66×10^3	-	1.0
5α-Pregnane-3α,15α,20β-triol	-	8.68×10^3	1.8

*The specific activity of the injected (4-^{14}C)-15α-hydroxyprogesterone was 3.66×10^5 dpm/μg.
-These metabolites were not isolated.

pregnan-20-one, while very small amounts of metabolites reduced at C-20 were isolated. In addition, a large proportion of the injected 15α-hydroxyprogesterone was excreted in the urine unchanged. These results suggest that the 15α-hydroxyl group inhibits the reduction of the 20-ketone and the Δ^4-3-keto group of the steroid nucleus. As shown in Table 63, some of the reduced metabolites of 15α-hydroxyprogesterone are 5α-steroids. The over-all ratio of the 5α to 5β reduced metabolites was found to be approximately 0.55.

Differences in the reduction of the Δ^4-double bond to the 5α and 5β forms *in vivo* which have been observed depend upon the nature of the substituent groups, particularly at carbon atoms 11 and 17. The *in vivo* catabolism of $C_{19}O_2$ steroids, such

as testosterone, androstenedione and dehydroisoandrosterone, in humans leads to the formation and excretion of the major urinary 17-ketosteroids with an overall ratio of 5α/5β reduced derivatives of approximately one to one (51). However, in vivo metabolism studies of adrenosterone, the C_{19}-steroid that possesses a ketone group at carbon 11, indicated a ratio of 5α- to 5β-reduced metabolites of 5.3 (52). Bradlow and Gallagher (53) studied the metabolism of 11β-hydroxyandrostenedione in a patient with congenital adrenal hyperplasia and found that the ratio of excreted 5α/5β metabolites was 4.8. It appears, therefore, that the oxygen substitution at carbon 11 of the $C_{19}O_2$ steroids orients the reduction of the Δ^4-double bond to the 5α configuration.

The generalization has been made that Δ^4-3-keto C_{21}-steroids are primarily reduced in vivo in humans to the 5β form (54). Verification of this rule was obtained in the studies on progesterone (55, 56), deoxycorticosterone (57), 21-deoxycortisone (58), cortisone (59), cortisol (60), and 17α-hydroxyprogesterone (61). Hirschmann et al (62) has commented on the apparent preponderance of 5α-metabolites in the urinary 16α-hydroxylated, C_{21}-steroids. That the 16α-hydroxyl group in the C_{21}-steroids influences the reduction in the A and B rings in favour of the 5α-metabolites was demonstrated by Ruse and Solomon (50) who found that the 5α-urinary metabolites of (4-^{14}C)-16α-hydroxyprogesterone injected into a pregnant female were present in amounts greater than would be expected for steroids of the C_{21} series. YoungLai and Solomon (63) studied the

metabolism of 16α-hydroxydehydroisoandrosterone in a pregnant female and in a normal male and found that the reduced urinary metabolites of this steroid were present in a 5α/5β ratio of approximately 1.0 and 3.7, respectively.

It seems, therefore, that the effect of the 15α-hydroxyl group on the reduction of the Δ^4-3-keto group of C_{21}-steroids is similar to the effect of the 16α-hydroxyl group in that the 5α/5β ratio of the urinary metabolites is increased. A comparison of the effects of the 15α- and 16α-hydroxyl substituents on the reduction of the Δ^4-3-keto and the 20-ketone groups of progesterone shows that both hydroxyl groups favour an increased 5α/5β ratio of the reduced urinary metabolites but that the 15α-hydroxyl group has a greater inhibitory effect on the reduction of the Δ^4-3-keto and the 20-ketone. In addition, reduction of the 20-ketone of 16α-hydroxyprogesterone favours the 20α-alcohols since only 20α-triols of 16α-hydroxyprogesterone were isolated (40). On the other hand, the only C_{20}-reduced metabolites of 15α-hydroxyprogesterone isolated in these studies were 5α-pregnane-3α,15α,20β-triol and 5β-pregnane-3α,15α,20β-triol.

A point of interest in these studies is that all the isolated metabolites of 15α-hydroxyprogesterone contained the 15-oxygen function. Calvin and Lieberman (64) demonstrated that a major metabolite of 16α—hydroxyprogesterone was isopregnanolone, a metabolite resulting from the removal of the 16α-hydroxyl group and the inversion of the 17β to the 17α side chain. The same authors found that 16-dehydropregnanolone was derived from 16α-hydroxyprogesterone and they suggested that a Δ^{16} compound

may be an intermediate in the formation of isopregnanolone. In these studies, no evidence for the removal of the 15-oxygen function of 15α-hydroxyprogesterone was obtained.

The urinary metabolites of (4-^{14}C)-15α-hydroxyprogesterone injected to a non-pregnant female are shown in Table 64. Again,

TABLE 64

Metabolites Isolated from the Urine of a Non-Pregnant Female After the Injection of (4-^{14}C)-15α-Hydroxyprogesterone*

Metabolite	Specific Activity (dpm/mg) Sulfate	Glucosiduronate	% Conversion of Injected Dose
15α-Hydroxyprogesterone	-	3.31×10^5	5.8
3α,15α-Dihydroxy-5α-pregnan-20-one	-	3.45×10^5	7.1
3α,15α-Dihydroxy-5β-pregnan-20-one	-	3.52×10^5	7.4
3β,15α-Dihydroxy-5β-pregnan-20-one	3.06×10^5	-	0.4
5α-Pregnane-3α,15α,20β-triol	-	3.35×10^5	2.3

*The specific activity of the injected (4-^{14}C)-15α-hydroxyprogesterone was 3.66×10^5 dpm/μg.
-These metabolites were not isolated.

3β,15α-dihydroxy-5β-pregnan-20-one was isolated from the sulfate fraction and 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3α,15α,20β-triol were isolated from the glucosiduronate fraction of the urine. The specific activities of all the metabolites were practically identical to the specific activity of the injected 15α-hydroxyprogesterone indicating that these steroids are not normall

excreted in the urine of the non-pregnant female.

It can be concluded from the results obtained in the studies described in this thesis that 15α-hydroxyprogesterone and its metabolites are present in pregnancy urine but not in the urine of the non-pregnant female. In addition, progesterone was converted to 15α-hydroxyprogesterone when infused into the fetus but such a conversion was not observed when progesterone was injected intravenously in the mother. These results indicate that 15α-hydroxylation of progesterone occurs in the fetoplacental unit and that urinary 15α-hydroxyprogesterone is of fetal origin.

The physiological significance of the 15α-hydroxysteroids formed during pregnancy remains to be elucidated. Since the uterotropic potency of 15α-hydroxyestradiol is only 1/1000 of that of estradiol benzoate in the immature female rabbit (3), 15α-hydroxylation of estrogens may be one of the mechanisms by which the developing fetus is protected against steroids with high biological activity (66). The role of fetal 15α-hydroxylation of progesterone is also presently not understood. Tweit and Kagawa (67) found that some 15—oxygenated derivatives of progesterone are capable of antagonizing the sodium-retaining and potassium-dissipating properties of deoxycorticosterone acetate in the adrenalectomized rat. Table 65 shows the relative activities of a number of pregnanes in terms of the dosage required for 50% inhibition of a standard dose (median effective dose) of deoxycorticosterone acetate. No significant antimineralocorticoid effects were noted for 15α-hydroxyprogesterone itself. However, the 15-oxo or 15β-OH derivatives and particularly those

TABLE 65

Relative Mineralocorticoid-Blocking Properties of Various 15—Oxygenated Derivatives of Progesterone in Adrenalectomized Rats

Steroid	Median Effective Dose* Subcutaneously	Orally
Progesterone	1.3	> 9.6
15α-Hydroxyprogesterone	> 2.4	> 2.4
15β-Hydroxyprogesterone	1.53	1.41
15-Ketoprogesterone	0.44	> 2.4
Pregn-1,4-diene-3,15,20-trione	0.23	0.25
Pregn-4,6-diene-3,15,20-trione	0.43	0.84
7α-Thioacetyl-15β-hydroxypregn-4-ene-3,20-dione	0.43	0.24
ALDACTONE	0.33	0.48

*Median effective dose = dose in mg per rat for 50% block of the urinary Na-K response to 12 μg of deoxycorticosterone acetate.

15-oxygenated derivatives possessing a Δ^1 or a Δ^6 double bond were effective as blocking agents for deoxycorticosterone acetate, sometimes these analogues being even more effective than aldactone (Table 65). Of considerable parallel interest was the observation that the 15-oxo or 15β-OH derivatives, while being effective as blocking agents, all failed to demonstrate progesterone-like changes in the uterine endometrium of immature rabbits primed with estradiol (67).

The secretion rate of aldosterone is elevated during pregnancy (68) and there is a progressive rise in the rate of

secretion with advancing pregnancy (69). Meyer et al (70) have shown that there is little or no alteration of the binding of aldosterone to plasma protein during pregnancy and the studies of Tait et al (71) demonstrate that the metabolic clearance rates of aldosterone in non-pregnant and pregnant women do not differ significantly. From studies of the excretion of urinary aldosterone in pregnant women with Addison's disease (72,73) and in adrenalectomized pregnant women (74,75), it was concluded that the hormone was elaborated by the maternal adrenals rather than by the fetus and placenta. The same conclusion was also reached from the studies of Bird et al (17) who investigated the metabolism of progesterone in the midterm previable fetus and could not detect aldosterone in any of the fetal tissues.

The mechanism for elevation of the aldosterone production during pregnancy is poorly understood. Landau et al (65,76) and Laidlaw et al (77) have demonstrated that progesterone has a natriuretic effect and have suggested that the natriuretic action of progesterone was due to an inhibition of aldosterone at the renal tubular level and that the competitive action of the two steroids may explain the elevated aldosterone secretion during pregnancy. This view was questioned by Watanabe et al (78) who found that when the fetus was dead and the placenta still in situ and elaborating progesterone, the aldosterone secretion rate had fallen to non-pregnant values. That the blood concentration of progesterone is still elevated in such subjects was demonstrated by Wiest (79). It can therefore be concluded that a viable fetus, and not the placental steroids alone, is mandatory for

an elevation of aldosterone secretion.

The amount of estriol excreted in the urine increases to very high levels in pregnancy, reaching a concentration of 60 mg/day or more during the last trimester of human gestation (80). Katz and Kappas (81) noted a transient natriuresis after the administration of large amounts of estriol and estradiol to human subjects. Earlier it was shown (82) that the oral administration of estrogens can elevate the aldosterone secretion.

Plasma renin in the mother is elevated during pregnancy (83). Furthermore renin activity has been found in the amniotic fluid (84) and this indicates that it may be elaborated by the placenta, but the site of its production is not known with certainty. Helmer and Judson (85) showed that there is an increase of renin substrate in the plasma of women with a normal pregnancy as well as in patients with toxemia of pregnancy. These authors suggest that the elevation in the concentration of renin substrate is due to the elevated estrogens elaborated during pregnancy. The result of an increase in renin substrate would be expressed as an increase in the amount of angiotensin elaborated and thus result in an increase in aldosterone secretion. Whether the estrogens do induce an increase in renin substrate remains to be demonstrated since it has been shown that estrogens are capable of increasing the aldosterone secretion rate (81) by a mechanism which has not yet been defined.

It can be seen from the foregoing discussion that the control of aldosterone secretion is complicated by many hormonal factors which operate during pregnancy and that the precise

mechanism of this stimulation is far from being understood at this time. However, present knowledge indicates that progesterone and estrogens may be involved in the alterations seen in aldosterone secretion rates in pregnant women. It was mentioned earlier that Tweit and Kagawa (67) have demonstrated that certain 15-oxygenated progesterones are capable of antagonizing the sodium-retaining and potassium-dissipating actions of deoxycorticosterone acetate in adrenalectomized rats. Although 15α-hydroxyprogesterone was not significantly active, compounds possessing a 15-ketone such as 15-ketoprogesterone and particularly 15-oxo progesterones with a Δ^1 or Δ^6 double bond were very active as antimineralocorticoid agents. Recently Stanczyk and Solomon (86) have shown that progesterone can be converted to 15-ketoprogesterone by rabbit liver homogenates. Whether the human fetal tissues can effect this conversion remains to be demonstrated and this problem is under investigation. The conversion of progesterone to 15-oxo derivatives possessing a Δ^1 bond can also be considered since such compounds are thought to be intermediates in the formation of estrogens. If the human fetus and/or the placenta are capable of converting 15α-hydroxyprogesterone to 15-ketoprogesterone or to 15-oxo derivatives with a Δ^1 or Δ^6 double bond, this might provide us with a mechanism by which a steroid elaborated by the fetoplacental unit can enter the maternal circulation (87) and compete with aldosterone thus inducing an increase in its rate of secretion. Although 15α-hydroxyprogesterone itself was not significantly effective as a mineralocorticoid antagonist in the adrenalectomized rat

(67), one preliminary observation has been provided in our laboratories to consider the possibility that this steroid might possess antimineralocorticoid properties in the human. When a single dose of 20 mg of 15α-hydroxyprogesterone was infused intravenously over a period of eight hours to a normal male who was on a low sodium diet, there was a 40% increase in the aldosterone secretion rate as compared to control values. Marked changes in electrolyte excretion and plasma renin concentrations were not observed in this study. It is therefore possible, although not as yet established, that 15α-hydroxyprogesterone made in the fetoplacental unit, or other members of this class of steroids, may play a role among the factors which stimulate and sustain the striking hyperaldosteronism which characterizes human pregnancy.

SUMMARY AND CONCLUSIONS

Labeled 15α-hydroxyprogesterone and 15α-hydroxyandrostenedione were prepared by the microbiological 15α-hydroxylation of the appropriate substrate with Penicillium (ATCC-11598) and in one instance with Colletotrichum linicola.

Three 15α-hydroxylated neutral steroids, namely, 15α-hydroxyprogesterone, 15α-hydroxyandrostenedione and 15α-hydroxytestosterone were isolated from urine pools obtained from subjects in the third trimester of pregnancy. When urine pools obtained from subjects with a variety of endocrine status were analyzed for 15α-hydroxyprogesterone, this steroid was only detected in the urine of subjects in the second and third trimester of pregnancy and it was not found in the urine of non-pregnant subjects or in the urine of newborn infants. These results indicated that 15α-hydroxyprogesterone is elaborated in the fetoplacental unit. In these studies sizable amounts of 16α-hydroxyprogesterone were isolated from the urine of a subject with congenital adrenal hyperplasia but 15α-hydroxyprogesterone was not detected in the urine of this subject.

The origin of 15α-hydroxyprogesterone was further investigated. In two studies ^{3}H-progesterone was introduced into the peritoneal cavity of the fetus at the time of amniocentesis for Rh incompatibility and at the same time ^{14}C-progesterone was injected in an antecubital vein of the mother. Following the injections urine was collected for 5 days and the urinary conjugates were hydrolyzed by solvolysis and then by β-glucu-

ronidase. From the glucosiduronate fraction ^{3}H-labeled 15α-hydroxyprogesterone was isolated but it contained insignificant amounts of ^{14}C demonstrating that 15α-hydroxylation of progesterone occurred in the fetoplacental unit. In a similar experiment, ^{3}H-progesterone was injected into the peritoneal cavity of the fetus and (4-^{14}C)-15α-hydroxyprogesterone was introduced in the maternal compartment. From the glucosiduronate fraction of the urine 15α-hydroxyprogesterone was isolated containing both ^{3}H and ^{14}C, and it was calculated that the minimal conversion of progesterone to 15α-hydroxyprogesterone in the fetoplacental unit was 0.08%.

After the intravenous injection of (7-^{3}H)-15α-hydroxyprogesterone and the oral administration of 800 mg of carrier steroid to a normal male the urinary conjugates were hydrolyzed with Glusulase. From the neutral extract the following metabolites were isolated and identified: 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3α,15α,20β-triol. In a second study, a total of 3.26 g of non-labeled 15α-hydroxyprogesterone were taken orally by four male volunteers and the urinary conjugates were hydrolyzed with Glusulase. In addition to the four metabolites described above, two new metabolites, namely, 3β,15α-dihydroxy-5β-pregnan-20-one and 5β-pregnane-3α,15α,20β-triol, were isolated and identified.

With the exception of 5β-pregnane-3α,15α,20β-triol, all of the metabolites mentioned above could be isolated from the urine of a pregnant subject given an intravenous injection of

(4-^{14}C)-15α-hydroxyprogesterone. Of these metabolites, 3β,15α-dihydroxy-5β-pregnan-20-one was isolated from the sulfate fraction and 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3α,15α,20β-triol were isolated from the glucosiduronate fraction of the urine. All of these metabolites have not hitherto been isolated from human urine. The major urinary metabolites of 15α-hydroxyprogesterone possess the Δ^4-3-keto and/or the 20-keto groups demonstrating that the 15α-hydroxyl group inhibits the reduction of the Δ^4-3-keto and 20-keto functions. A large proportion of the isolated metabolites had the 3α,5α configuration indicating that the 15α-hydroxyl group has an influence on the reduction of the A/B ring. In addition only 20β-hydroxylated metabolites were isolated in contrast to the studies done with 16α-hydroxyprogesterone where the only 20-reduced metabolites isolated were the 20α-triols (50). In all the studies described in this thesis 15α-hydroxyprogesterone was found only in the glucosiduronate fraction of the urine.

Following the intravenous injection of (4-^{14}C)-15α-hydroxyprogesterone to a non-pregnant female, all the metabolites isolated from the urine of the pregnant female were again isolated but their specific activities were identical to the specific activity of the injected 15α-hydroxyprogesterone. These results demonstrate that the metabolites of 15α-hydroxyprogesterone are not excreted in the urine of the non-pregnant female.

CLAIMS TO ORIGINAL RESEARCH

1. Microbiological methods were devised for the preparation of labeled 15α-hydroxysteroids.
2. Three 15α-hydroxylated neutral steroids, namely, 15α-hydroxyprogesterone (88), 15α-hydroxyandrostenedione and 15α-hydroxytestosterone were isolated from human late pregnancy urine. These steroids have not hitherto been reported as naturally occurring substances.
3. Among a large number of urine pools examined, 15α-hydroxyprogesterone was isolated only from the urine of subjects in the second and third trimester of pregnancy. This steroid was not detected in the urine of non-pregnant females and the urine of the newborn infant.
4. Following the simultaneous injection of ^{3}H-progesterone to the peritoneal cavity of the fetus at the time of amniocentesis and of ^{14}C-progesterone in an antecubital vein of the mother, 15α-hydroxyprogesterone isolated from the urine contained ^{3}H but insignificant amounts of ^{14}C. These results demonstrated that 15α-hydroxylation of progesterone occurred in the fetoplacental unit and that the mother elaborated little, if any, 15α-hydroxyprogesterone.
5. It was demonstrated that the conversion of progesterone to 15α-hydroxyprogesterone in the fetoplacental unit was approximately 0.08%.
6. The production rate of 15α-hydroxyprogesterone in two females in late pregnancy was calculated to be about 80-90 μg/day.

7. Sizable amounts of 16α-hydroxyprogesterone were isolated from the urine of a subject with congenital adrenal hyperplasia. This is the first time that this steroid has been isolated from the urine of such subjects.
8. The following urinary metabolites of 15α-hydroxyprogesterone were isolated and identified: 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 3β,15α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,15α,20β-triol and 5β-pregnane-3α,15α,20β-triol.
9. When labeled 15α-hydroxyprogesterone was injected intravenously to a subject in the third trimester of pregnancy, 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 3β,15α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3α,15α,20β-triol were isolated from the urine and their specific activities were 25-40 times lower than the specific activity of the injected 15α-hydroxyprogesterone. These results demonstrated that all the metabolites isolated are normally excreted in the urine of subjects in late pregnancy. All of these steroids have not hitherto been isolated from human urine.
10. From the nature of the isolated urinary metabolites of 15α-hydroxyprogesterone it was concluded that the 15α-hydroxyl group inhibits the reduction of the Δ^4-3-keto and 20-keto groups and has an influence on the reduction of the A/B ring of the steroid nucleus giving rise to a larger proportion of 5α metabolites than would be expected for steroids

in the C-21 series.

11. When labeled 15α-hydroxyprogesterone was administered intravenously to a non-pregnant female, all the metabolites isolated from the urine of the pregnant female were again detected in the urine but their specific activities were identical to the specific activity of the injected 15α-hydroxyprogesterone demonstrating that these steroids are not normally excreted in the urine of the non-pregnant female.

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