METABOLISM OF 16α-HYDROXYSTEROIDS IN MAN

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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
androstenedione	androst-4-ene-3,17-dione
testosterone	17β-hydroxyandrost-4-en-3-one
androstanetriol	5α-androstane-3α,16α,17β-triol
etiocholanetriol	5β-androstane-3α,16α,17β-triol
16α-hydroxydehydroisoandrosterone	3β,16α-dihydroxyandrost-5-en-17-one
16α-hydroxyandrostenedione	16α-hydroxyandrost-4-ene-3,17-dione
16α-hydroxytestosterone	16α,17β-dihydroxyandrost-4-en-3-one
16α-hydroxyandrosterone	3α,16α-dihydroxy-5α-androstan-17-one
16α-hydroxyetiocholanolone	3α,16α-dihydroxy-5β-androstan-17-one
pregnenolone	3β-hydroxypregn-5-en-20-one
progesterone	pregn-4-ene-3,20-dione
deoxycorticosterone	21-hydroxypregn-4-ene-3,20-dione
hydrocortisone	11β,17α,21-trihydroxypregn-4-ene-3,20-dione
aldosterone	11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al
isopregnanolone	3α-hydroxy-17α-pregnan-20-one
11-dehydrocorticosterone	21-hydroxypregn-4-ene-3,11,20-trione
16α-hydroxypregnenolone	3β,16α-dihydroxypregn-5-en-20-one
16α-hydroxyprogesterone	16α-hydroxypregn-4-ene-3,20-dione
16-dehydroprogesterone	pregn-4,16-diene-3,20-dione
16-dehydropregnanolone	3α-hydroxy-5β-pregn-16-en-20-one

cholesterol	3β-hydroxycholest-5-ene
estrone	3-hydroxyestra-1,3,5(10)-trien-17-one
estradiol	estra-1,3,5(10)-triene-3,17β-diol
estriol	estra-1,3,5(10)-triene-3,16α,17β-triol
16α-hydroxyestrone	3,16α-dihydroxyestra-1,3,5(10)-trien-17-one
sulfate	3β-yl sulfate
Δ^4-isomerase	3-ketosteroid Δ^4-isomerase
HCG	human chorionic gonadotropin
SD	standard deviation
N	Normality, that is, the equivalent weight of a substance in 1 litre of solution
g	acceleration due to gravity
g	gram
µg	microgram
mg	milligram
µC	microcurie
mC	millicurie
mM	millimole
ml	milliliter
dpm	disintegrations per minute
mp	melting point
mmp	mixed melting point
ppm	parts per million
^oC	degrees Centigrade
Rf	the ratio of the velocity of the substance under consideration to the velocity of the mobile phase in a chromatographic system.

CONTENTS

INTRODUCTION

It was in 1930 that the first 16α-hydroxysteroid, namely estriol, was isolated from human pregnancy urine by Marrian (1) and Doisy et al (2). In the ensuing years, a number of 16α-hydroxysteroids, both neutral and phenolic, have been isolated from various sources. A large quantity of 16α-hydroxylated metabolites have been isolated from normal human urine, from the urine of individuals with various diseases and from human pregnancy urine. Neher et al (3) isolated 3α,16α-dihydroxy-5α-pregnan-20-one and 3α,16α-dihydroxy-5β-pregnan-20-one from the urine of a subject with the salt-losing form of congenital adrenal hyperplasia. In initial experiments with rats it was reported that these steroids were responsible for salt loss. When 3β,16α-dihydroxy-5α-pregnan-20-one was tested in humans it did not produce any salt loss (4) although it was previously found to be natriuretic in rats.

The search for a direct physiological action of 16α-hydroxysteroids has not been too successful. In recent years it has become evident that fetal tissues are capable of introducing a 16α-hydroxyl group on a variety of preformed steroid precursors. It is now established that the 16α-hydroxylated C-19 steroids originating in the human fetus are precursors of estriol formed in the placenta (5). A small number of studies concerning the metabolism of 16α-hydroxysteroids has been recorded in the literature (6,7). Before discussing the present investigations, current knowledge regarding 16α-hydroxysteroids will be reviewed.

Isolation of 16α-hydroxysteroids from urine

Although 16α-hydroxysteroids such as 5α-androstane-3α,16α-diol and its 16β-epimer (8) and 5α-pregnane-3β,16α,20β-triol (9-13) have been found in pregnant mares' urine the majority of isolation studies have been carried out on human urine.

In Table I are listed the various 16α-hydroxysteroids which have been isolated from human urine. As can be seen from the data in this Table, the most abundant 16α-hydroxysteroids isolated were those containing 19 and 21 carbon atoms. A number of investigators have isolated 16α-hydroxydehydroisoandrosterone, 16α-hydroxypregnenolone and Hirschmann's triol. It is interesting to note that these three 3β-hydroxy-5-ene steroids have been isolated from the urine of newborn infants, a reflection of the activity of the fetal zone of the adrenal and an apparent lack of 3β-hydroxysteroid dehydrogenase.

Recently, Lipsett et al (40) reported a secretion of 11 mg of 16α-hydroxydehydroisoandrosterone per day by a 67-year old man with metastatic cell tumor of the testis. Bongiovanni (23) reported that 16α-hydroxypregnenolone was secreted to an extent of 3.0-4.5 mg per day by a patient with the adrenogenital syndrome associated with a deficiency of the 3β-hydroxysteroid dehydrogenase. Androst-5-ene-3β,16α,17β-triol was first isolated from the urine of a boy with adrenocortical carcinoma (31). Ruse and Solomon (33) have isolated 500 μg of crystalline 16α-hydroxyprogesterone from the urine of a pregnant subject in the third trimester.

Fukushima et al (37) isolated an unusual steroid, pregn-5-ene-3α,16α,20α-triol, from the urine of a patient with widespread adrenocortical carcinoma, the first report of the isolation of a 3α-hydroxy-5-ene steroid. The authors suggested that in patients with adrenocortical hyperplasia the major secretory product may be 16α-hydroxypregnenolone which could yield the 3α-hydroxysteroid from the intermediate 16α-hydroxypregn-5-ene-3,20-dione, together with an inhibition of the Δ^4-isomerase enzyme by the 16α-hydroxyl group. In 1963, Fukushima and Gallagher (38) reported the secretion of 4.0 mg of pregn-5-ene-3α,16α,20α-triol per day in the urine of a patient with "non-functioning" adrenocortical carcinoma.

TABLE 1

16α-Hydroxysteroids Isolated from Human Urine

Steroid	Clinical Status	Reference
C-18		
16α-hydroxyestrone	pregnant female	14-16
estriol	normal female	17,18
	pregnant female	1,2
C-19		
16α-hydroxydehydroisoandrosterone	normal male	19-22
	normal infant	23-26
	premature infant	27
	adrenal tumor	28
	adrenogenital syndrome	23
androst-5-ene-3β,16α,17β-triol (Hirschmann's triol)	normal male	20,22,29
	normal female	29
	female pseudohermaphrodite	30
	premature infant	27
	adrenal tumor	31
5α-androstane-3α,16α,17β-triol	unspecified illnesses	32
5β-androstane-3α,16α,17β-triol	unspecified illnesses	32
3β,7α,16α-trihydroxyandrost-5-en-17-one	adrenal tumor	28
3β,16α-dihydroxyandrost-5-ene-7,17-dione	adrenal tumor	28
C-21		
16α-hydroxypregnenolone	normal male	22
	normal infant	24,25
	premature infant	27
	pregnant female	33
	adrenal tumor	23
16α-hydroxyprogesterone	pregnant female	33
3α,16α-dihydroxy-5α-pregnan-20-one	adrenal tumor	3
3α,16α-dihydroxy-5β-pregnan-20-one	adrenal tumor	3
pregn-5-ene-3β,16α,20α-triol	normal male	22,34
	adrenal tumor	35-38
pregn-5-ene-3α,16α,20α-triol	adrenal tumor	37,38
5α-pregnane-3α,16α,20α-triol	pregnant female	7,39
5β-pregnane-3α,16α,20α-triol	pregnant female	7,39

Conjugated 16α-hydroxysteroids have also been isolated from human urine. When dehydroisoandrosterone sulfate was given intravenously to a normal male 16α-hydroxydehydroisoandrosterone sulfate and androst-5-ene-3β,16α,17β-triol sulfate were isolated from the urine (41-43). Roberts et al (44) demonstrated that cholesterol sulfate can form urinary 16α-hydroxydehydroisoandrosterone sulfate when given to a female patient with massive adrenocortical carcinoma.

Isolation of 16α-hydroxysteroids from tissues

Steroids having a 16α-hydroxyl group have been isolated from a variety of mammalian tissues. In 1931, Browne reported the isolation of estriol from human placenta (45). Subsequently, estriol has been isolated from the following human tissues: the placenta (46) in amounts of 315 μg per kg of wet weight (47), cord blood (48), pregnancy plasma (49), ovaries of young women obtained soon after death (50), ovaries during the luteal phase (51), corpora lutea of pregnant women in the first trimester (52), meconium in the order of 2.5-4.7 μg per 800 g of tissue (53,54) and fetal tissues (48). Diczfalusy and Magnusson (48) reported an estriol concentration of 346 μg per kg of fetal liver tissue, 94 μg per liter of amniotic fluid and 696 μg per kg of cord tissue.

Neher and Wettstein in 1960 (55) isolated 16α-hydroxytestosterone from the testicular tissue of young bulls. This steroid was again isolated from extracts of human placenta (56) and was reported to be present in placental tissue incubations in amounts of 2.0 μg per 100 g per hour (57).

In 1964, Magendantz and Ryan (58) and Colas et al (59,60) described the isolation of 16α-hydroxydehydroisoandrosterone from umbilical artery and vein blood. In these studies (59) the titer of 16α-hydroxydehydroisoandrosterone

was reported to be 147 ± 42 (SD) μg per 100 ml of plasma in arterial blood and 144 ± 35 (SD) μg per 100 ml of plasma in venous blood. These findings suggested that 16α-hydroxydehydroisoandrosterone was transferred from the fetus to the placenta. This was not substantiated by examination of plasma samples obtained in the course of Caesarean sections (60). Very recently, Easterling et al (61) found 16α-hydroxydehydroisoandrosterone sulfate with an average concentration of 87 μg per 100 ml of plasma in cord venous blood and 110 μg per 100 ml of plasma in cord arterial blood, whereas the values for the unconjugated steroid were 17 μg per 100 ml and 24.5 μg per 100 ml respectively. None of these compounds could be detected in cord blood when anencephalic fetuses were present.

Hog adrenal glands have been found to be a good source of 3β,16α-dihydroxy-5α-pregnan-20-one (62), 3α,16α-dihydroxy-5α-pregnan-20-one (3) and 16α-hydroxyprogesterone (63). Zander et al (64-66) have reported the isolation of 16α-hydroxyprogesterone from extracts of human corpora lutea (0.29 μg per g of tissue) and from placental blood obtained from umbilical cord (0.8 μg per 100 ml plasma). The presence of 16α-hydroxypregnenolone in umbilical cord blood was reported by Eberlein (67).

Steroid 16α-hydroxylases

The ability of a number of animal tissues to introduce the 16α-hydroxyl group on a preformed steroid precursor has been studied extensively. In Table 2 are shown the various species involved and the different substrates used to demonstrate 16α-hydroxylation. Various types of experimental designs have been employed to demonstrate these conversions. These have ranged from perfusion of the previable human fetus to incubations of different tissue preparations with the appropriate precursor. It is well established that the human fetal liver and adrenals possess very active steroid 16α-hydroxylases.

TABLE 2

Steroids Used to Demonstrate 16α-Hydroxylase Activity in Tissues

Precursors	Species	Test System	Reference
C-18			
Estrone	Cattle	adrenal minces	68
	Man	perfused fetus	69
		term placenta	70
Estradiol	Laying hen	liver slices	71
	Rats	liver preparations	72-75
	Man	fetal liver	76
		fetal adrenal	77
		adult ovaries	78
		term placenta	70,79
C-19			
Testosterone	Rats	liver microsomes	80,81
		testes homogenates	82
	Dogs	perfused liver	83
	Man	fetal adrenal	84
		adult ovaries	85
Dehydroisoandrosterone	Rats	liver slices	86,87
	Rhesus monkey	liver microsomes	87
	Rabbit	liver slices	88
	Man	fetal liver	87,89
		fetal adrenal	90
		perfused fetus	91
C-21			
Pregnenolone	Man	perfused fetus	92,93
		fetal liver	94
		newborn adrenal	95
		adult liver	94,96
		adult adrenal	95
Progesterone	Hogs	ovaries	97
		adrenal homogenates	98
	Cattle	fetal ovaries	99
		adrenal homogenates	63
	Man	fetal adrenals	92,93,100-105
		fetal testes	106
		fetal kidneys	92,93,105
		placental homogenates	107,108
		newborn adrenals	95
		adult testes	109,110
		adult ovaries	111

All the examples cited in Table 2 have involved the use of normal tissues. Tissues obtained from subjects with a variety of pathological conditions have often provided material for the demonstration of 16α-hydroxylations. Hyperplastic adrenocortical tissue possesses 16α-hydroxylase activity for pregnenolone (112) and progesterone (113-115). Villee (103) and Adadevoh et al (116) have reported the conversion of hydrocortisone and progesterone respectively to the corresponding 16α-hydroxylated products using minced adrenal slices from patients with Cushing's syndrome. A feminizing adrenal tumor was also found to be capable of converting pregnenolone and progesterone to 16α-hydroxyprogesterone (117).

It was first demonstrated in 1963 by Acevedo et al (118) that human fetal testes can convert progesterone to 16α-hydroxyandrostenedione and pregnenolone to Hirschmann's triol. Testes from patients with testicular feminization also possessed the ability to 16α-hydroxylate progesterone (119-122). Recently, Axelrod and Goldzieher (123) reported the in vitro conversion of pregnenolone to 16α-hydroxytestosterone by sclerocystic ovaries from a 37-year old woman with the adrenogenital syndrome. Tumor tissue from a dysgenetic gonad removed from an 18-year old girl was reported to possess progesterone 16α-hydroxylase activity (124).

Although perfusion of the human term placenta with estrone (70) and estradiol (70,79) in the presence of HCG resulted in the formation of estriol, this conversion was not observed with perfused midterm placentas (125). However, when dehydroisoandrosterone and dehydroisoandrosterone sulfate were administered simultaneously into a uterine artery, estriol could be detected in the placenta (126). Very recently, Kirschner et al (127) demonstrated that dehydroisoandrosterone sulfate was a better precursor than estrone sulfate for the formation of estriol in the feto-placental circulation at mid-pregnancy.

In the same paper the authors found the reverse to be true when estrone sulfate and dehydroisoandrosterone sulfate were injected into the antecubital vein of the mother. In this experiment it was concluded that the urinary estriol was derived mainly from the estrone sulfate. Baulieu and Dray (128) had previously found that dehydroisoandrosterone sulfate was a better precursor than androstenedione or testosterone of urinary estriol in pregnant and normal women.

Formation of estriol from 16α-hydroxysteroids

The investigations of Ryan have been instrumental in elucidating many aspects of estriol biosynthesis in the human placenta. In 1958 he demonstrated that the 10,000 g supernatant fraction of homogenized placenta containing microsomes and soluble enzymes was capable of converting androst-5-ene-3β,16α,17β triol to estriol in 12% yield (129). Using the same test system he later showed that 16α-hydroxyandrostenedione and 16α-hydroxytestosterone can also be aromatized to estriol in good yield (130). Recently, Magendantz and Ryan (58) and Colas et al (131) demonstrated the conversion of 16α-hydroxydehydroisoandrosterone to estriol by placental microsomes, 16α-hydroxyandrostenedione being formed as an intermediate. Varangot et al (132,133) have reported that the perfused term placenta was capable of aromatizing 16α-hydroxyandrostenedione and 16α-hydroxytestosterone and this capacity was enhanced in the presence of HCG.

The formation of estriol from 16α-hydroxylated C-21 precursors has been reported by Kadis (97,134) using 16α-hydroxyprogesterone and minces of sow ovary. More recently, Pierrepoint et al (135) have demonstrated the conversion of tritiated 16α-hydroxypregnenolone to estriol by homogenates and minces of a feminizing Leydig cell tumor of the testis.

Siiteri and Macdonald (5,136) have studied the precursor role of 16α-hydroxydehydroisoandrosterone in the formation of urinary estriol in pregnant subjects. They calculated that as much as 55% of the injected 16α-hydroxydehydroisoandrosterone may be converted into estriol (5). The authors suggested that the huge amounts of estriol produced during pregnancy may arise from such a neutral intermediate.

Metabolism of 16α-hydroxysteroids

Except for a few isolated studies the metabolism of 16α-hydroxysteroids has not been studied to any great extent. In 1962 Calvin and Lieberman (6) published the first paper on the metabolism of a 16α-hydroxylated steroid in man. They demonstrated the conversion of labelled 16α-hydroxyprogesterone to urinary isopregnanolone and 16-dehydropregnanolone and suggested that the isopregnanolone may arise through an intermediate such as 16-dehydroprogesterone. Very recently, Ruse and Solomon (7) demonstrated that labelled 16α-hydroxyprogesterone can be converted to urinary isopregnanolone, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,16α,20α-triol and 5β-pregnane-3α,16α,20α-triol. Siiteri and Macdonald (5) reported that 16α-hydroxydehydroisoandrosterone was metabolized to urinary Hirschmann's triol and estriol during pregnancy. Other metabolites were not investigated in this study. The meagre evidence regarding the metabolism of 16α-hydroxysteroids in man and the fact that 16α-hydroxydehydroisoandrosterone was found in high titers in umbilical vein (58,59,61), led to the working hypothesis that some of the 16α-hydroxydehydroisoandrosterone may enter the maternal circulation to be further metabolized.

Biological significance of 16α-hydroxysteroids

The biological role of 16α-hydroxysteroids is not well established.

In 1958 Bernstein (137) stated that the presence of the 16α-hydroxy group lowers the salt-retaining activity of corticoids without affecting their glucocorticoid activity. Several other investigators have observed this natriuretic property of different 16α-hydroxylated corticoids using various experimental animals (62,138-141) but this effect could not be demonstrated in man (4). Studies on adrenalectomized rats showed that the 16α-hydroxylation of progesterone reduces its ability to antagonize the electrolyte effects of aldosterone whereas 16α-hydroxypregnenolone had no effect (142). It was demonstrated that 16α-hydroxyhydrocortisone possessed appreciable activity in a number of animal assays (glycogen, thymus involution, anti-inflammatory and Karnofsky egg embryo tests) but no electrolyte activity could be detected (138). Synthetic 16α-hydroxysteroids have been used as anti-inflammatory agents (140,143).

Recent investigations by several groups have implicated 16α-hydroxylated C-19 steroids as major precursors of estriol during pregnancy (5,58,144,145). In the studies (5,58) it was observed that 16α-hydroxylated C-19 steroids were converted to estriol in good yield. Moreover, after the antecubital injection of labelled C-19 steroids, namely, dehydroisoandrosterone sulfate (5,127,128), androstenedione (128), testosterone (128) and 16α-hydroxydehydroisoandrosterone (5,136) to pregnant subjects, urinary estriol containing a high proportion of the injected label could be isolated. Thus it seems that the primary function of 16α-hydroxysteroids lies in the formation of estriol.

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.

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

Benzene (Analytical grade, Mallinckrodt) was distilled over potassium hydroxide pellets prior to use. Dry benzene was prepared by distilling benzene with phosphorus pentoxide in a closed system. The dry reagent was stored in a desiccator.

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

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.

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

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

The reagents listed below were all distilled prior to use without any additional treatment:

Acetone (analytical grade, Mallinckrodt)

n-Butanol (Fisher certified)

Cyclohexane (practical grade, Distillation Products Ltd.)

Ethyl acetate (Fisher certified)

Ethylene dichloride (Fisher certified)

Heptane (Fisher certified)

n-Hexane (Fisher certified)

Iso-octane (practical grade, Distillation Products Ltd.)

Isopropyl ether (Fisher certified)

Methanol (Fisher certified)

Methylene chloride (Fisher certified)

Methyl cellosolve (Fisher certified)

Skellysolve B(Skelly Oil Co.)

Toluene (Fisher certified)

Tertiary butanol (practical grade, Distillation Products Industries)

Steroids

The following steroids were used as standards:

Androstanetriol (a gift of Dr. D. Fukushima, Montefiore Hospital, New York.)

Androstenedione (Syntex)

Dehydroisoandrosterone (Syntex)

Dehydroisoandrosterone sulfate (a gift of Dr. R. Deghenghi, Ayerst Laboratories, Montreal.)

Deoxycorticosterone (Schering, Berlin)

Estriol (Schering, Berlin)

Etiocholanetriol (a gift of Dr. J. Schneider, Jefferson Medical College, Pennsylvania.)

3α,16α-Diacetoxyandrostan-17-one (a gift of Dr. D. Fukushima, Montefiore Hospital, New York.)

3α,16α-Diacetoxyetiocholan-17-one (a gift of Dr. D. Fukushima, Montefiore

Hospital, New York)

16α-Hydroxyandrostenedione (a gift of Dr. P. Diassi, E.R. Squibb and Co., New Brunswick, N.J.)

16α-Hydroxydehydroisoandrosterone (a gift of Dr. S. Bernstein, Lederle Laboratories, Pearl River, New York.)

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 recrystallization.

Radioactive steroids were obtained from New England Nuclear Corp., Boston, Massachusetts.

Special Reagents

Blue Tetrazolium (Fisher certified): A 0.2% (w/v) aqueous solution was mixed with 10% (w/v) sodium hydroxide and 60% (v/v) aqueous methanol in the proportions of 1:1.5:1.5 immediately before use.

Methylene Blue reagent: 250 mg Methylene Blue (Fisher certified), 50 g anhydrous sodium sulfate and 10 ml concentrated sulfuric acid were mixed with one litre of distilled water prior to use.

Oertel's reagent: Two parts of concentrated sulfuric acid was added cautiously to one part of absolute ethanol and mixed thoroughly immediately before use.

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.

Reagents for the Zimmerman reaction: (a) 2% (w/v) m-dinitrobenzene (Fisher certified, recrystallized from ethanol before use) in absolute ethanol.

(b) 2.5 N potassium hydroxide in absolute ethanol.

Alumina (200 mesh; Harshaw Chemical Co., Cleveland, Ohio) was washed and deactivated according to the procedure described by Solomon et al (146). 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 72-120 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). Compounds 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. 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. Two models of the Packard Tri-Carb liquid scintillation spectrometer were used for counting. Only one instrument was used in any one experiment.

When one isotope was counted, the settings of the instrument were as follows: In the Model 3002 the amplifier gain of both channels was set at 60% and pulse height discriminators at 50 to infinity. At these settings ^{3}H was counted with an efficiency of 39% and ^{14}C with an efficiency of 91%. In the presence of methanol the efficiency of counting was 19% and 83% 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; and gain on the green channel was set at 40% with the discriminators at 700 to infinity. Efficiency of counting at these settings was 33% for ^{3}H and 62% 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 ∞. Efficiency of counting at these settings was 15% for ^{3}H and 53% for ^{14}C.

The efficiency of counting was calculated with the use of ^{3}H and ^{14}C labelled 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 until a constant weight was obtained. Aliquots of ^{3}H or ^{14}C hexadecane (dissolved in absolute ethanol) were then transferred to the vials and dried over low heat under 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.

Single label counting in the Model 4322 was accomplished using a gain in the red channel of 50% with the discriminators set at 50 to 1000, while the gain in the green channel was set at 8% with discriminators at 50 to 1000. The blue channel was maintained at a gain of 2% and discriminators at 400 to ∞ throughout. At these settings the efficiency of counting ^{3}H and ^{14}C was 38% and 87% respectively. In the presence of methanol the corresponding efficiencies were 20% and 78% respectively. In simultaneous counting without methanol ^{3}H was measured in the red channel at 40% gain and discriminator settings of 40 and 500, and ^{14}C in the green channel at 6% gain and discriminator settings of 140 and 1000. Efficiency of counting was about 32% for ^{3}H and 64% for ^{14}C. In the presence of methanol ^{3}H was counted in the red channel at 62% gain and discriminator settings of 50 and 550, and ^{14}C in the green channel at 10% gain and discriminator settings of 160 and 1000. Efficiency for ^{3}H and ^{14}C was about 17% and 55% respectively.

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 (147) as modified by Ulick (148). The following equations were used:-

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

and $$^{14}C = N_2 - N_{1a}$$

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. With the Model 3002 the optimum conditions for double label counting in the absence of methanol were obtained with an "a" ratio of approximately 0.006 and a "b" ratio of 3.17; in the presence of methanol, the "a" and "b" ratios were approximately 0.017 and 4.08 respectively. The corresponding "a" and "b" ratios in the Model 4322 were 0.004 and 5.67 without methanol in the counting vial, and 0.005 and 3.96 with methanol. All counts were converted directly to dpm.

Counts of single labelled compounds were corrected for quenching with the use of ^{3}H and ^{14}C labelled toluene as internal standard. Vials were recounted after addition of a known amount of radioactivity in 0.1 ml or 0.2 ml 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 ^{14}C and ^{3}H 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.).

Infrared Spectroscopy

Infrared spectra were obtained with the use of a Perkin-Elmer Model 221 Spectrophotometer. Samples were prepared as described by Roberts et al (149) 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. In some instances only micro amounts (20-50 μg) were available for infrared analysis and these were examined in KBr with the aid of a Perkin-Elmer 6X microsampling unit (Model 186-0011).

Mass Spectroscopy

Analysis by mass spectroscopy was perfomed through the courtesy of Dr. C. Djerassi, Department of Chemistry, Stanford University, California.

Nuclear Magnetic Resonance Spectroscopy

Analysis by nuclear magnetic resonance spectroscopy was kindly done by Dr. J. Fishman, Institute for Steroid Research, Montefiore Hospital, New York.

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 2 ml per g of alumina were collected. Steroids were eluted with increasing concentrations of benzene in Skellysolve B or with increasing concentrations of ether or 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 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 and stationary phase was added slowly. Except where noted, 0.5 ml of stationary phase per g of Celite was used. The mixture was transferred to a glass chromatographic column and the Celite packed tightly with a Martin packer (150).

When possible, samples were dissolved in mobile phase and a concentrated 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. In a few instances the extracts were dissolved in small amounts of stationary phase and applied directly to the column without any resulting distortions in the elution pattern. Mobile phase was delivered from a glass reservoir attached to the top of the column. The effluent was collected in tubes using an automatic fraction collector (Buchler Instruments, N.J.). Hold-back volumes (HBV) of columns were determined by measuring the volume of solvent required to elute the dye, Sudan IV, as described by Johnson (151) 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.

Column fractions were analysed by measuring the radioactivity of suitable aliquots from each fraction. In one experiment to be described the analysis for steroid sulfates in column fractions was carried out by means of the Methylene Blue reaction as modified by Roy (152). Aliquots of samples to be analysed were dried, dissolved in 1 ml of 50% (v/v) Methylene Blue reagent and extracted with 5 ml of chloroform. The steroid sulfate formed a complex with the Methylene Blue reagent and this complex was extracted into the chloroform layer giving a blue color. The density of the color was measured at 650 mμ in a Coleman Junior Spectrophotometer.

Paper Chromatography: Strips of Whatman No. 1 or No. 3 MM filter paper 15 x 57 cm were used throughout. Chromatography on paper was accomplished by either one of two methods. In the first method, using the procedure described by Bush (153), 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. In the second method, according to Zaffaroni et al (154) the paper was impregnated with stationary phase by dipping the paper in 50% solution of stationary phase in methanol as described by Savard (155). The paper was then blotted twice just prior to the application of the samples. No equilibration was necessary for such systems.

For reversed phase paper chromatography, acetylated papers, Whatman No. AC 81 (W.R. Balston Ltd., England) and S and S No.2496 (Schleicher and Schnell Co., Keene, N.H.) were used. After application of the sample the paper was equilibrated for a minimum of four hours with the organic phase before development with aqueous phase.

Ultraviolet absorbing steroids were located on paper with the aid of

a viewing box (Chromato-Vue, Ultraviolet Products Inc., San Gabriel, Calif.). Other 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 (156). Steroids having an α-ketol group at C_{16-17} were detected by the use of the Blue Tetrazolium reaction as described by Burton et al (157). Strips of paper to be tested were dipped in the Blue Tetrazolium reagent and blotted. A purple color against a pink background indicated the presence of an α-ketol. Steroids having a Δ^5-3β-hydroxyl configuration were detected by means of the Oertel reaction (158). The paper strip was placed on a glass plate and the reagent was poured gently over it. The appearance of a straw color which later turned pink indicated the presence of a Δ^5-3β-hydroxyl function. Steroids bearing the 17-keto group were detected on paper by means of the Zimmerman reaction as described by Savard (155). The paper strip was first dipped in m-dinitrobenzene, blotted, and then dipped in ethanolic potassium hydroxide, and heated to 100°C for 1-2 minutes. A purple color against a pale yellow background indicated the presence of a 17-ketosteroid. Steroid sulfates were detected on paper by means of the Methylene Blue reaction described by Roy (152). The strip of paper containing the sulfate was passed several times through a 50% solution of Methylene Blue reagent, blotted dry and then washed thoroughly by dipping the paper several times in chloroform. The complex formed between the steroid sulfate and Methylene Blue was soluble in the chloroform thereby giving a white spot which appeared against a blue background. Radioactive steroids were located with either of two chromatogram strip-scanners,

a Vanguard Model 880 or a Packard Model 7200.

Areas of paper containing steroid to be eluted were cut in small squares, covered with methanol and allowed to stand for 18—24 hours. After filtering the solvent, the paper squares were extracted twice with additional amounts of methanol. The total methanol extract was evaporated in vacuo. When propylene glycol was present the residue after evaporation of methanol was dissolved in ethyl acetate and washed twice with 1/5 volume water to remove any traces of 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 the initial developing solvent was transferred to a glass column which was tapped vigorously to ensure even settling of the support and in order to remove air bubbles. The sample to be chromatographed was applied to the top of the column in 1% solution of ethanol in methylene chloride and the column was developed with increasing concentrations of ethanol in methylene chloride. The effluent from the columns 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. Aliquots of fractions were removed at suitable intervals for the determination of radioactivity and individual fractions from each column were combined according to the plot of radioactivity versus fraction number.

Small silica gel columns were also used for the further purification of the steroids eluted after paper chromatography. These were prepared in the manner described above but here fractions of 2 ml 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 20 x 20 cm glass plates to a depth of 1mm, using a Research Specialities 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 10 cm line, 2 cm from one edge. Chromatograms were developed by ascending solvent flow in small glass tanks with glass covers.

Steroids were located on the thin layers by examination in U V light or by spraying with phosphomolybdic acid as previously described for paper chromatograms. When necessary, radioactivity was located by counting the eluates of 1 cm^2 sections of the silica gel from the length of the plate. Immediately after chromatography the silica gel to be eluted was scraped off the plate with a spatula 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 extraction was repeated twice, after which the combined ethyl acetate was evaporated at 40°C.

Solvent systems used in all of the chromatographic separations to be described in the thesis are shown in Table 3.

Preparation of Steroids for Injection

Whenever possible labelled steroids were stored in benzene:methanol (4:1) solution 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 dissolved in 0.5 ml of absolute ethanol. Immediately before injection 10 ml of sterile isotonic saline was added to the vials

TABLE 3

Solvent Systems Used in Chromatography

System	Solvents
A	Benzene:cyclohexane:methanol:water (1:2:3:3)
B	Benzene:n-butanol:methanol:water (10:0.6:3:3)
C	Cyclohexane:methanol:water (25:22:3)
D	n-Hexane:ethyl acetate:methanol:water (10:10:13:7)
E	Heptane:methyl cellosolve (1:1)
F	Iso-octane:ethyl acetate:methanol:water (2:1:1:1)
G	Iso-octane:t-butanol:ammonium hydroxide:water (14:18:1:19)
H	Iso-octane:t-butanol:ammonium hydroxide:water (12:20:1:19)
J	Iso-octane:t-butanol:ammonium hydroxide:water (10:20:1:19)
K	Iso-octane:t-butanol:methanol:water (20:8:3:9)
L	Benzene:cyclohexane:ethyl acetate:methanol:water (8:1:1:2:8)
M	Iso-octane:methanol:water (10:9:1)
N	Benzene:cyclohexane (1:1) - propylene glycol
O	Methylene chloride-ethylene glycol
P	Toluene-propylene glycol
Q	Iso-octane:toluene:methanol:water (5:5:7:3)
R	Iso-octane:toluene:methanol:water (3:5:4:1)
S	Isopropyl ether:t-butanol:ammonium hydroxide: water (6:4:1:9)
T	Toluene:ethyl acetate:methanol:water (19:1:13:7)
U	Toluene:n-butanol:ammonium hydroxide:water (5:5:1:9)
W	Skellysolve B:methanol:water (10:9:1)
Y	Toluene:ethyl acetate:methanol:water (9:1:6:4)

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. In the experiments to be reported, insignificant amounts of radioactivity were recovered from the syringes, needles and vials. This small number (less than 1%) was subtracted from the radioactivity originally prepared for the injection.

Hydrolysis of Urinary Conjugates

In the initial experiments reported, an ether extraction was carried out on each 24-hour urine sample prior to extraction of the conjugates. Insignificant amounts of radioactivity were found in every case. The later experiments were therefore carried out without this initial extraction. 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 (159) was employed. Each day's urine was adjusted to pH 1 with concentrated sulfuric acid. After addition of sodium chloride, 20% (w/v), the urine was extracted in five identical aliquots 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 in the dark at 37°C overnight. 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 5% $NaHCO_3$ to remove pigments and acids, and with water until the water washes were neutral. Stronger alkalis were avoided in order to preserve the α-ketols expected as urinary metabolites. 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.

The residual aqueous phase plus the alkali and water washes from the above extraction contained the steroid glucosiduronidates. 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 60 mg of powdered β-glucuronidase preparation (Baylove Chemicals, Musselburgh, Scotland) per 100 ml of solution. Methylene chloride, 10 ml, was added to the solution as a 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 glucosiduronidates and also those steroids excreted as double conjugates with sulfuric and glucuronic acids.

In one experiment simultaneous enzymatic hydrolysis of sulfates and glucosiduronidates was accomplished with the use of Glusulase (Endo Laboratories, New Jersey), 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 and 0.5 ml of the enzyme mixture per 100 ml of urine were added. 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 a glass stoppered tube, and left in the dark at room temperature for about 18 hours. Steroids acetylated with labelled acetic anhydride were dissolved in one part of pyridine and three parts of a 10% solution of acetic anhydride in benzene and incubated at 37°C overnight. A greater than 0.2 molar excess of acetic anhydride per mol of steroid was used throughout.

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. Benzene:methanol (1:1) was added and they were evaporated. The procedure was repeated several times until the odor of pyridine had disappeared. In the second method, the reaction mixture was transferred to ice water containing 5% of 6N sulfuric acid, and the acetate 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*.

Hydrogenation

Hydrogenation of a Δ^5-double bond using 3α-acetoxyandrost-5-en-17-one and 3α,16α-diacetoxyandrost-5-en-17β-ol was accomplished by the method described by Fukushima et al (37), using an apparatus kindly provided by Dr. R. Deghenghi, Ayerst Laboratories, Montreal. The steroid acetate was dissolved in 10 ml ethyl acetate, to which was added 100 mg

platinum oxide and a few drops of 70% perchloric acid. The mixture was then shaken in a hydrogen atmosphere under slight pressure for 20 hours. At the end of the hydrogenation the residue was filtered and the filtrate evaporated in vacuo. The residue was purified by chromatography on a small alumina column.

Hydrolysis of acetates: Steroid acetates were dissolved in a minimum volume of 0.4M methanolic potassium hydroxide. After standing for 3 hours at room temperature the methanol was removed on the flash evaporator and 50 ml of distilled water was added. Then the mixture was extracted with ethyl acetate. For the extraction of trihydroxysteroids sodium chloride was added to the aqueous layer to a concentration of 20% before extraction. The ethyl acetate extract was then washed until neutral with distilled water, dried over Na_2SO_4 and evaporated in vacuo.

Sodium borohydride reduction: Reduction with $NaBH_4$ was performed by the method described by Norymberski and Wood (160) 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 10-15 minutes. 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. When polar steroids were present NaCl was added to the aqueous layer to make a 20% solution before extraction. Then the organic phase was washed with 0.1N sodium hydroxide, water, dried over Na_2SO_4 and evaporated in vacuo.

Dichlorodicyanobenzoquinone Oxidation and Preparation of 16α-acetoxytestosterone: A derivative of 16α-acetoxyandrostenedione 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 modifi-

cation by Ruse and Solomon (33) of the original method of Burn et al (161). A total of 28 mg of 16α-acetoxyandrostenedione was reduced with $NaBH_4$ as described above. The products of reduction were dissolved in 2 ml of freshly distilled dioxane contained in a glass stoppered tube to which was added 22.2 mg of DDQ. The reaction mixture was left in the dark at room temperature for 24 hours and the hydroquinone formed was filtered and the filtrate evaporated to dryness. The residue was dissolved in ethyl acetate and the organic phase was washed with 0.5N NaOH until pigment removal was complete, and then with water, dried over Na_2SO_4, filtered and evaporated in vacuo. The residue, 26.6 mg, was chromatographed on a 2 g silica gel column, starting with 20% ether in benzene. Elution with 40% and 80% ether in benzene yielded 23.4 mg of crystalline material. Crystallization from methanol-ether gave 12.8 mg of coarse needles, mp 175-177°C, which were UV positive when a solution of the crystals was applied on paper.

A comparison of the infrared spectrum (CS_2) of the product with the original compound before reduction indicated the retention of the acetate and the presence of a Δ^4-3-ketone function, the loss of the 17-ketone group and the appearance of a hydroxyl group. We were not able to compare the synthesized compound with an authenticated standard. However, it is known (160) that reduction with $NaBH_4$ gives mainly the hydroxyl group in the β-configuration. Also, under the conditions employed, only α,β-unsaturated alcohols are oxidized by DDQ. With this evidence we can with some confidence assign to the product the structure, 16α-acetoxytestosterone.

Preparation of Pyridinium sulfate:

Reagents. Chloroform was washed three times with concentrated sul-

furic acid until colorless, with 1N sodium hydroxide until alkaline and then with water until neutral. The chloroform was then dried over calcium chloride, filtered, and distilled immediately before use. Concentrated sulfuric acid was dried over phosphorus pentoxide in a desiccator under reduced pressure.

The actual preparation of pyridinium sulfate was carried out according to the method described by Levitz (162). Pyridine and chloroform were mixed in the ratio of 5 moles of chloroform to 1 mole of pyridine. Thirty parts of this mixture were added with vigorous stirring to 1 part of concentrated sulfuric acid. A white flocculent precipitate formed, which was centrifuged, washed three times with chloroform, dried in vacuo and stored in a stoppered container in a desiccator.

Recovery of Steroids from Counting Vials and KBr discs

In some instances material taken for counting and for infrared analysis (KBr) had to be recovered in order to continue with an identification or purification process (163). 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.

Recovery of steroid from KBr discs was accomplished by crushing the disc into a powder and dissolving the powder in water. The mixture was then extracted with ethyl acetate, and the ethyl acetate was dried over sodium sulfate and evaporated in vacuo.

Standardization of Labelled Acetic Anhydride

A stock solution of 1-^{14}C-acetic anhydride (Batch #1), specific activity

10 mC/mM, was prepared by diluting 1 mC (10.2 mg) of the reagent in 0.04 ml of benzene with 0.22 ml of acetic anhydride and 2.0 ml of dry benzene. Two serial dilutions (10-fold) with 10% acetic anhydride in benzene afforded solutions 1 and 2. Another stock solution of 1-^{14}C-acetic anhydride (Batch # 2), specific activity 1 mC/mM was diluted to make a 20% solution in benzene. Two dilutions of this solution (25-fold and 125-fold) with 20% acetic anhydride in benzene gave solutions "A" and "B". A total of 204 mg of ^{3}H-acetic anhydride (Batch # 3) of specific activity 100 mC/mM was diluted with 5 ml of dry benzene and another dilution was made by dissolving 0.25 ml with 1.5 ml of non-labelled acetic anhydride. The specific activity of the acetic anhydride in each solution except those of Batch # 1 was determined by the acetylation of deoxycorticosterone (DOC) and the measurement of the specific activity of the resulting acetate (DOCA). The solutions of Batch # 1 were standardized with 16α-hydroxydehydroisoandrosterone (16D). After acetylation of known amounts of non-labelled steroid the products were chromatographed on alumina columns prior to crystallization. Constant specific activity of each acetate was achieved as shown in Table 4.

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

Following initial chromatography on a column of silica gel the material within each radioactive peak was further purified and partially identified by chromatography on a Celite column and/or paper. To facilitate crystallization of the individual steroids at this stage, they were percolated through small silica gel columns as previously described. For the determination of the endogenous specific activities, one of four of the following methods was used:

a) The isolated metabolite was crystallized directly from an appropriate solvent mixture until the final specific activities of the crystals and

TABLE 4

Standardization of Labelled Acetic Anhydride*

	Crystallization	Specific Activity (dpm/mg 16 DA or DOCA) Crystals	Mother Liquors
Batch # 1			
Solution 1	1	1.19×10^5	1.18×10^5
	2	1.18	1.21
	3	1.21	1.20
Solution 2	1	1.18×10^4	1.19×10^4
	2	1.19	1.19
Batch # 2			
Solution "A"	1	1.12×10^5	1.05×10^5
	2	1.04	1.30
	3	1.17	1.07
	4	1.17	1.15
Solution "B"	1	2.24×10^4	2.00×10^4
	2	2.28	2.25
	3	2.28	2.29
Solution "C"	1	4.62×10^3	4.62×10^3
	2	4.76	4.73
Batch # 3	1	2.01×10^6	2.19×10^6
	2	2.13	2.10

*The solutions of Batch # 1 were standardized against 16α-hydroxydehydroisoandrosterone while those of Batches # 2 and # 3 were standardized against deoxycorticosterone. The acetic anhydride of Batches # 1 and # 2 was labelled with ^{14}C while that of Batch # 3 was labelled with ^{3}H.

mother liquor did not differ by more than 10%. Following each crystallization 0.600 mg to 1 mg 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 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 150 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) Labelled acetic anhydride was used to determine the specific activities of certain urinary metabolites using the double isotope derivative principles described by Kliman and Peterson (164). The isolated compound containing ^{3}H was acetylated with one of the standardized solutions of 1-^{14}C-acetic anhydride and the product mixed with the appropriate carrier acetate. The mixture was then chromatographed on a small alumina or silica gel column and crystallized until the $^{3}H/^{14}C$ ratios of the crystals and mother liquors were constant. The specific activity of the isolated steroid was calculated according to the expression:

$$S.A. = \frac{a\ b\ d\ n}{C}$$

where S.A. = specific activity of the isolated metabolite
a = specific activity of DOCA (dpm ^{14}C/mg)
b = molecular weight of DOCA
C = molecular weight of the metabolite
d = $^{3}H/^{14}C$ ratio of the acetylated metabolite
n = number of acetylatable groups.

c) In the instances where the isolated metabolite contained both ^{3}H and ^{14}C the specific activity was determined indirectly by the method of Siiteri (165). The specific activity with respect to one of the isotopes ..

of such a metabolite was determined as follows: Separate aliquots of the purified metabolite were acetylated with both non-radioactive and radioactive acetic anhydride. The choice of the label and specific activity of the acetic anhydride to be used depended on the magnitude of the $^{3}H/^{14}C$ ratio and weight of the purified metabolite to be acetylated. Following purification and crystallization of each type of acetate with carrier as described above, an $^{3}H/^{14}C$ ratio, corrected for the isotope content of the unacetylated metabolite was calculated from the following expressions:

a) when ^{14}C-acetic anhydride was used -

$$(T/C) = \frac{(T/C)_1}{1-\frac{(T/C)_1}{(T/C)_o}}$$

where (T/C) = corrected $^{3}H/^{14}C$ ratio

$(T/C)_1$ = $^{3}H/^{14}C$ ratio of acetate prepared from ^{14}C-acetic anhydride

$(T/C)_o$ = $^{3}H/^{14}C$ ratio of acetate prepared from non-labelled acetic anhydride.

This corrected ratio was then substituted in the expression described above for determination of the specific activity of isolated metabolites.

b) when ^{3}H-acetic anhydride was used -

$$(T/C)_c = (T/C)_t - (T/C)_a$$

where $(T/C)_c$ = corrected $^{3}H/^{14}C$ ratio

$(T/C)_t$ = $^{3}H/^{14}C$ ratio of acetate prepared from ^{3}H-acetic anhydride

$(T/C)_a$ = $^{3}H/^{14}C$ ratio of acetate prepared from non-labelled acetic anhydride

This corrected ratio was then substituted in the following formula:

$$S.A._c = \frac{d\ m\ n}{RM}$$

where $S.A._c$ = specific activity of the isolated metabolite with respect to ^{14}C.

d = specific activity of DOCA

m = molecular weight of DOCA

n = number of acetylatable groups

R = $^{3}H/^{14}C$ ratio

M = molecular weight of the unacetylated metabolite.

From the value obtained the specific activity of the metabolite with respect to ^{3}H was then determined by the use of the final $^{3}H/^{14}C$ ratio obtained after the aliquot was acetylated with non-labelled acetic anhydride.

d) Isotope Ratio Procedure: In some instances no carrier steroid or its acetate was available for crystallizations. Following acetylation as in b) and c) described above the products were chromatographed on paper or on a variety of columns. When the peak of radioactivity was located on columns or on paper, aliquots were taken from the middle of the peak and from both the proximal and distal ends. The $^{3}H/^{14}C$ ratio was determined from the three aliquots and the remaining material was rechromatographed until the $^{3}H/^{14}C$ ratio was constant in two successive chromatographic procedures. If after these chromatographic steps the $^{3}H/^{14}C$ ratio was constant, the metabolite was subjected to the following procedure in order to assess its radiochemical purity. To this product was then added authentic labelled acetate of known purified radioactive content. These authentic acetates were prepared from metabolites isolated in one experiment where the identity of each metabolite and a high degree of purity were established. The amount and type of labelled acetate added depended on the $^{3}H/^{14}C$ of the isolated metabolite after acetylation. If the $^{3}H/^{14}C$ ratio was low, then labelled acetate containing ^{3}H was added. On the other hand, when the $^{3}H/^{14}C$ was high, labelled acetate with both ^{3}H and ^{14}C was added. This procedure was adopted to obtain optimal counting conditions. The mixture was then chromatographed until a constant $^{3}H/^{14}C$ ratio was obtained. If this final ratio was the same as that predicted after the addition of the labelled acetate, then the identity of the metabolite is established. The specific activity of each metabolite thus identified was determined from the $^{3}H/^{14}C$ ratio obtained prior to the addition of authentic labelled acetate. This technique was

developed because of the unavailability of large amounts of carrier steroid for reverse isotope dilution analyses and will be referred to as the Isotope Ratio Procedure.

Preparation of Labelled Substrates

Labelled 16α-hydroxylated substrates were prepared by the incubation of appropriate labelled substrates with a strain of Streptomyces roseochromogenus (ATCC 3347) kindly supplied by Dr. C. Vezina, Ayerst Laboratories, Montreal. This organism is capable of hydroxylating the steroid nucleus at the 16α-position (166).

(7α-^{3}H)-16α-hydroxydehydroisoandrosterone (Lot#1). Streptomyces roseochromogenus was maintained on slants of Gould's agar. A two to four week growth containing spores, in 0.01% of aqueous Dupanol (sodium lauryl sulfate) was transferred as a suspension to a 250 ml Erlenmeyer flask containing 50 ml of sterile E2B medium which consisted of 30 g of glucose, 20 g of soybean meal, 2.2 g of hydrogenated soybean oil and 2.5 g of calcium carbonate, all in one litre 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 Streptomyces roseochromogenus. After 72 hours a 10% transfer of the growing organism was made to a second flask containing 50 ml of E2B medium. Tritium labelled dehydroisoandrosterone was added in 2x0.2 ml of acetone to the second flask, and the mixture incubated for 48-66 hours as before.

The substrate, 1 mC of (7α-^{3}H)-dehydroisoandrosterone in 0.17 mg was used as purchased. Prior to use it was diluted with 6.76 mg of carrier dehydroisoandrosterone to give a theoretical specific activity of 3.17×10^8 dpm/mg.

After incubation, the medium was extracted with methylene chloride,

1x100 ml and 2x75 ml. The combined methylene chloride extract was evaporated under vacuum, and the residue was dissolved in 15 ml methylene chloride to which was added 300 ml methanol. A white flocculent precipitate appeared and the mixture was stored at 4°C. overnight before filtering through a medium porosity sintered glass funnel. The filtrate was dried in vacuo to yield 57.1 mg of residue which contained 1.31×10^9 dpm. This residue was chromatographed on two thin-layer plates in system ethyl acetate:n-hexane (2:1) with dehydroisoandrosterone and 16α-hydroxydehydroisoandrosterone as standards. The standards were located on the thin layer plates with phosphomolybdic acid and the area corresponding to 16α-hydroxydehydroisoandrosterone was eluted. The eluate which weighed 9.0 mg and contained 8.45×10^8 dpm was chromatographed on paper in system Q for 4 hours. Elution of the peak of radioactivity corresponding to standard 16α-hydroxydehydroisoandrosterone gave 5.7 mg of a residue containing 6.88×10^8 dpm. This was mixed with 9.0 mg carrier and was chromatographed on a 1 g silica gel column using ethanol in methylene chloride. Elution with 2% ethanol in methylene chloride yielded 13.6 mg of crystalline material containing 6.81×10^8 dpm. This material was crystallized once from acetone-ether yielding 7.5 mg crystals with a specific activity of 5.05×10^7 dpm/mg. The mother liquor had a specific activity of 4.86×10^7 dpm/mg. The crystals were used in the first two studies to be described. The yield of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone was 33%.

An aliquot of the crystalline material containing 3.11×10^5 dpm was mixed with 30.3 mg of carrier steroid and the mixture crystallized as shown in Table 5.

(7α-^{3}H)-16α-Hydroxydehydroisoandrosterone (Lot #2). Another lot of labelled 16α-hydroxydehydroisoandrosterone of higher specific activity was prepared from 1 mC of (7α-^{3}H)-dehydroisoandrosterone (0.177 mg, contain-

TABLE 5

Proof of Radiochemical Purity of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone

Crystallization	Specific Activity (dpm/mg)	
	Crystals	Mother Liquor
1	9,900	11,000
2	10,100	10,500
3	10,400	10,200
Calculated*	10,300	

*A total of 311,000 dpm was mixed with 30.3 mg of carrier 16α-hydroxydehydroisoandrosterone prior to crystallization.

ing 1.98×10^9 dpm). The purity of the tritiated steroid was proved by reverse isotope dilution analysis of an aliquot as shown in Table 6. The remaining

TABLE 6

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

Crystallization	Solvent	Specific Activity (dpm/mg)	
		Crystals	Mother liquor
1	Acetone-Skellysolve B	1240	1290
2	Ether-Skellysolve B	1280	1320
3	Methanol-ether	1230	1250
	Calculated*	1290	

*A total of 50,660 dpm was mixed with 38.5 mg of carrier prior to crystallization.

labelled steroid was mixed with 5.69 mg of carrier and incubated with Streptomyces roseochromogenus as previously described.

The final methylene chloride extract obtained after incubation weighed

30.2 mg and contained 1.61×10^9 dpm. It was chromatographed on two thin-layer plates in system ethyl acetate:n-hexane (2:1), and the area corresponding in mobility to 16α-hydroxydehydroisoandrosterone was eluted to yield a residue which weighed 3.72 mg and contained 1.13×10^9 dpm. Chromatography on paper in system Q for 7 hours gave one radioactive zone with the mobility of 16α-hydroxydehydroisoandrosterone. It was eluted and re-chromatographed on paper in system P for 20 hours. Again, one radioactive zone was obtained at an average distance of 10.4 cm which had the same mobility as standard 16α-hydroxydehydroisoandrosterone. Elution of this zone with methanol afforded 7.5 mg of residue which contained 9.74×10^8 dpm. It was percolated through a 1 g silica gel column and elution with 2% and 3% ethanol in methylene chloride yielded 3.9 mg of crystals which contained 9.39×10^8 dpm. An aliquot of this material was mixed with carrier and crystallized to constant specific activity as shown in Table 7. The yield of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone

TABLE 7

Proof of Radiochemical Purity of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	4860	4700
2	4700	4690
3	4890	4780
Calculated*	5060	

*A total of 187,840 dpm was mixed with 37.1 mg carrier prior to crystallization.

was 48% and the calculated specific activity based on the carrier added and the altered molecular weight was 3.19×10^8 dpm/mg.

(4-^{14}C)-16α-Hydroxyandrostenedione. When purchased 100 μC of (4-^{14}C)-androstenedione was contained in 0.633 mg and an aliquot was checked for purity by reverse isotope dilution as shown in Table 8. Under the conditions

TABLE 8

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

Crystallization	Specific Activity (dpm/mg) Crystals	Mother Liquor
1	550	520
2	540	530
3	550	540
Calculated*	530	

*A total of 41,880 dpm was mixed with 79.1 mg of carrier and crystallized.

used for counting this amount contained 2.37x10^8 dpm. It was mixed with 4.66 mg of carrier and incubated with Streptomyces roseochromogenus using the identical conditions as described for hydroxylating dehydroisoandrosterone.

The methylene chloride extract obtained after extraction (31.0 mg and 2.16x10^8 dpm) was chromatographed on 2 thin layer plates in system ethyl acetate:n-hexane (2:1). The UV positive area corresponding to 16α-hydroxyandrostenedione standard was eluted to yield 4.87 mg of residue which contained 1.60x10^8 dpm. This eluate was chromatographed on paper in system P for five hours and the radioactive zone with a mobility of 23.7 cm, corresponding to standard, was eluted to yield 7.0 mg of residue containing 1.30 x10^8 dpm. Further chromatography of this residue on paper in system N for 29 hours and elution of the radioactive UV positive area which had a mobility of 14.8 cm, gave 5.0 mg of residue containing 1.23x10^8 dpm. This was percola-

ted through a 1 g silica gel column. Elution with 2% ethanol in methylene chloride gave 2.96 mg of crystalline material containing 1.20×10^8 dpm. The yield of (4-^{14}C)-16α-hydroxyandrostenedione was 51%. The calculated specific activity of the 16α-hydroxyandrostenedione was 4.28×10^7 dpm/mg. An aliquot containing 35,870 dpm was mixed with 41.3 mg carrier and the mixture was crystallized to constant specific activity as shown in Table 9.

TABLE 9

Proof of Radiochemical Purity of (4-^{14}C)-16α-Hydroxyandrostenedione

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	820	890
2	840	860
3	820	850
Calculated*	870	

*A total of 35,870 dpm of (4-^{14}C)-16α-hydroxyandrostenedione was mixed with 41.3 mg carrier and crystallized.

(4-^{14}C)-Dehydroisoandrosterone-3-sulfate. The preparation of steroid sulfates was accomplished by the method described by Fieser (167). One ml of pyridine in a glass-stoppered tube was cooled in an ice-bath and to this was added 0.1 ml of p-chlorosulfonic acid with constant shaking. To this mixture was added 50 μC of (4-^{14}C)-dehydroisoandrosterone, specific activity 42 mC/mM, (used as purchased), using 4x0.1 ml of pyridine. This mixture was heated on a steam bath until it became clear and then was allowed to stand for half an hour. Solvents were removed in vacuo, and the residue was dissolved in 10 ml of 1N ammonium hydroxide, extracted with 2 x 10 ml of ether,

and with 4 x 10 ml of n-butanol. The butanol extract was dried under vacuum and the residue was chromatographed on paper in system S for 8½ hours. One symmetrical zone of radioactivity was obtained corresponding in mobility to dehydroisoandrosterone-3-sulfate run alongside. Elution with methanol gave 5.2×10^7 dpm representing a yield of 47%. An aliquot containing 18,860 dpm was mixed with 9.6 mg of carrier and the mixture crystallized as shown in Table 10.

TABLE 10

Proof of Radiochemical Purity of (4-^{14}C)-Dehydroisoandrosterone-3-sulfate

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	2090	1850
2	1920	2070
Calculated*	1960	

*A total of 18,860 dpm of (4-^{14}C)-dehydroisoandrosterone-3-sulfate was mixed with 9.6 mg carrier and recrystallized.

(7α-^{3}H)-16α-Hydroxydehydroisoandrosterone-3-sulfate (Lot #1). The procedure used for the hydroxylation of dehydroisoandrosterone-3-sulfate was the same as that described for the preparation of the unconjugated steroid, except that the substrate was dissolved in water before transferring it to the incubation medium. The substrate, 1 mC of (7α-^{3}H)-dehydroisoandrosterone-3-sulfate (used as purchased) was mixed with 15 mg of carrier and the mixture was incubated with the Streptomyces roseochromogenus as described. After incubation, the medium was extracted with ether and then by the method

of Edwards, Kellie and Wade (168). The aqueous layer was adjusted to a concentration of 50% with ammonium sulfate and then extracted three times with equal volumes of ether-ethanol (3:1). The organic extract was dried in vacuo to yield 81 mg of residue containing 5.5×10^8 dpm. It was chromatographed on four Whatman No.3 MM sheets of paper in system S for 10 hours to yield two main radioactive zones, AI and AII, with average mobilities of 7.4 cm and 31.0 cm respectively. The eluate of AI which contained 2.2×10^8 dpm and weighed 19.0 mg was chromatographed on paper in system U for 6 hours. One main radioactive zone was obtained with a mobility of 24.9 cm. Elution with methanol gave 3.3×10^7 dpm. An aliquot containing 677,500 dpm was mixed with 20 mg of carrier 16α-hydroxydehydroisoandrosterone-3-sulfate (the preparation of which will be described later) and the mixture was crystallized. Constant specific activity was achieved after the third crystallization and again after solvolysis of the conjugate as shown in Table 11. The yield of

TABLE 11

Proof of Radiochemical Purity of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone-3-sulfate

	Specific Activity (dpm/mg)			
	16α-Hydroxydehydroisoandrosterone-3-sulfate		16α-Hydroxydehydroisoandrosterone	
Crystallization	Crystals	Mother Liquor	Crystals	Mother Liquor
1	32,120	37,290	6,580	5,510
2	32,580	33,100	6,460	6,420
3	32,690	33,050	6,360	6,400
Calculated*	33,880		6,480	

*A total of 677,500 dpm of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone-3-sulfate was mixed with 20 mg of carrier and crystallized from methanol and methanol-ether. The material present in the third crystals and mother liquor was combined and solvolysed. The unconjugated steroid containing 181,800 dpm was mixed with 25.4 mg of carrier 16α-hydroxydehydroisoandrosterone and the mixture was purified on a silica gel column, to yield 26.3 mg of crystals containing 170,670 dpm. The calculated specific activities were based on these values.

(7α-^{3}H)-16α-hydroxydehydroisoandrosterone-3-sulfate was too low to be used, but it demonstrated that a steroid conjugate could be hydroxylated at the 16α-position by micro-organisms.

(7α-^{3}H)-16α-Hydroxydehydroisoandrosterone-3-sulfate (Lot #2). (7α-^{3}H)-Dehydroisoandrosterone (5 mC, specific activity 1.62 Curies/mM) was checked for purity by chromatography of a small aliquot on paper in system W for 7 hours. One symmetrical peak of radioactivity was obtained corresponding in mobility to dehydroisoandrosterone. The substrate which contained 1.1×10^{10} dpm and weighed 0.885 mg was sulfated as previously described. Chromatography of the butanol extract on paper in system S gave one radioactive zone corresponding in mobility to dehydroisoandrosterone-3-sulfate and contained 9.3×10^9 dpm on elution. An aliquot containing 115,200 dpm was mixed with 22.5 mg of carrier and the mixture was crystallized twice from methanol-ether and methanol. as shown in Table 12. The remainder of the (7α-^{3}H)-dehydroisoandrosterone-3-sulfate was incubated and extracted as described above. From the ether-ethanol extract a residue was obtained which weighed 52.7 mg and

TABLE 12

Proof of Radiochemical Purity of (7α-^{3}H)-Dehydroisoandrosterone-3-sulfate

Crystallization	Specific Activity (dpm/mg)	
	Crystals	Mother Liquor
1	5380	5220
2	4970	5120
Calculated*	5120	

*A total of 115,200 dpm of (7α-^{3}H)-dehydroisoandrosterone-3-sulfate was mixed with 22.5 mg carrier and the mixture was crystallized.

contained 6.2×10^9 dpm. It was chromatographed on a 50 g Celite column using system H and a plot of radioactivity versus fraction number is shown in Figure I. At fraction 60 the mobile phase was changed to system J. The material in fractions 73 to 97 were combined to yield 38.1 mg of a residue containing 5.03×10^9 dpm. This residue was chromatographed on two Whatman No.3 MM papers in system U for 6½ hours. One symmetrical zone of radioactivity was obtained having the same mobility as 16α-hydroxydehydroisoandrosterone-3-sulfate run along side. Elution with methanol gave 4.1×10^9 dpm and this represented a yield of 44% based on the radioactivity in dehydroisoandrosterone-3-sulfate. An aliquot containing 4.1×10^5 dpm was solvolysed in the same manner as that described for the solvolysis of urinary sulfates. The product was mixed with 48.3 mg of carrier and the mixture chromatographed on a 4 g silica gel column. Elution with 2% ethanol in methylene chloride gave 46.7 mg of crystalline material containing 271,900 dpm. This material was crystallized to constant specific activity as shown in Table 13.

TABLE 13

Proof of Radiochemical Purity of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	5900	9150
2	5840	6050
3	5790	5590
Calculated*	5840	

*After chromatography on a silica gel column 46.7 mg of crystalline material containing 2.72×10^5 dpm was obtained.

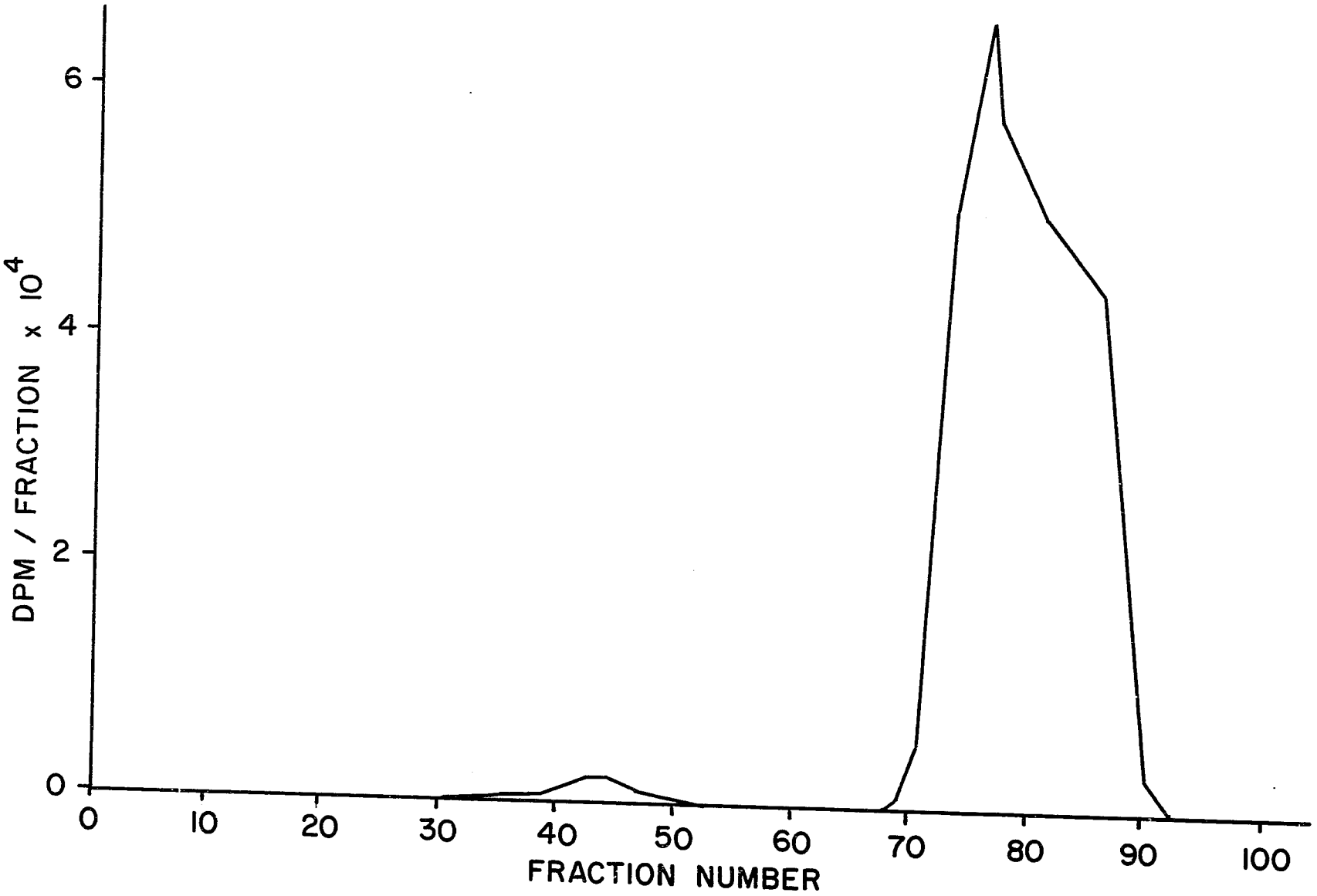

FIG. I. Celite column chromatography of ether-ethanol extract obtained after the incubation of (7α-^{3}H)-dehydroisoandrosterone sulfate with Streptomyces roseochromogenus.

A second aliquot containing 5.7×10^5 dpm was mixed with 0.4 mg of carrier 16α-hydroxydehydroisoandrosterone-3-sulfate and the mixture was chromatographed on paper in system U. One symmetrical peak of radioactivity was obtained with an Rf of 0.56 and the material within this peak gave positive reactions with phosphomolybdic acid, Blue Tetrazolium and the Methylene Blue reagent.

16α-Hydroxydehydroisoandrosterone-3-sulfate. Non-radioactive dehydroisoandrosterone-3-sulfate (1.5 g) was 16α-hydroxylated using the same procedure as the one described previously for the synthesis of the labelled 16α-hydroxydehydroisoandrosterone-3-sulfate. After incubation, the medium was filtered through a bed of Celite and extracted three times with equal volumes of n-butanol. The combined butanol layers were evaporated under vacuum and the residue obtained was dissolved in water and extracted twice with equal volumes of ether. To the aqueous layer was then added ammonium sulfate to a concentration of 50% and the solution was extracted three times with ether-ethanol (3:1) according to the method of Edwards, Kellie and Wade (167). The organic layer was dried in vacuo to yield an oily residue weighing 25 g which was chromatographed on a 700 g Celite column using system G. The rate of elution was 45 ml per hour and a plot of Methylene Blue positive material in every 10th fraction versus fraction number is shown in Figure 2. Pool I contained dehydroisoandrosterone-3-sulfate. At fraction 1800 the mobile phase was changed to system J and another Pool, II, containing Methylene Blue positive material was eluted. After combining the material contained within peak II 870 mg of a gummy residue was obtained. It was reacted with 0.3M pyridinium sulfate solution (w/v) according to the method of McKenna and Norymberski (169) to form the pyridinium salt which was extracted into chloro-

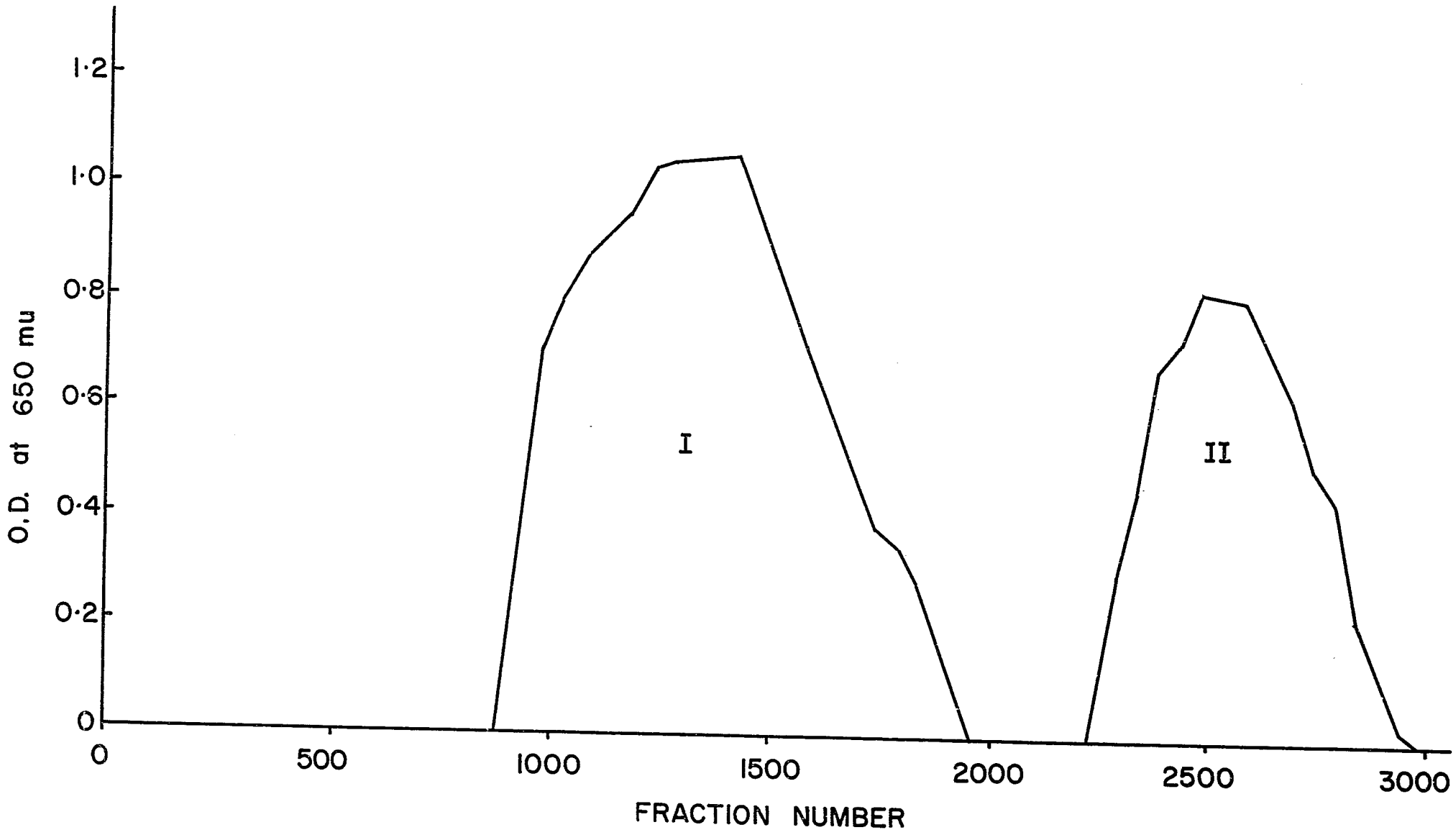

FIG. 2. Celite column chromatography of ether-ethanol extract obtained after the incubation of dehydroisoandrosterone sulfate with Streptomyces roseochromogenus.

form. The chloroform extract was dried in vacuo, and the residue obtained was dissolved in a 1.2 molar excess of ammonium hydroxide and extracted with n-butanol. The organic extract was dried under vacuum to yield 150 mg of amorphous powder which was percolated through a 15 g silica gel column. Elution with 25% - 35% ethanol in methylene chloride gave 91 mg of white residue which was crystallized three times from methanol and methanol-ether to give 26.5 mg of crystals. The infrared spectrum (KBr) showed sharp distinct bands at 1740 cm^{-1}, 1067 cm^{-1}, 1015 cm^{-1}, 983 cm^{-1}, 955 cm^{-1}, 863 cm^{-1}, 825 cm^{-1}, (triplet) 735 cm^{-1}, and broad bands at 3400 cm^{-1}, 3150 cm^{-1}, 1275-1200 cm^{-1}. A 2.1 mg aliquot was solvolysed using the procedure of Jacobsohn and Lieberman (159). The solvolysed product was chromatographed on a small silica gel column to yield 1.7 mg of crystals which were eluted with 2% ethanol in methylene chloride. This material was crystallized twice from acetone and acetone-Skellysolve B. The infrared spectrum (KBr) of the second crystals was identical to that of 16α-hydroxydehydroisoandrosterone. The crystals had a mp of 178-180°C and a mp of 179-180°C with standard 16α-hydroxydehydroisoandrosterone; reported mp 177-181°C (19).

EXPERIMENTAL SECTION AND RESULTS

EXPERIMENT I. Metabolism of 16α-hydroxydehydroisoandrosterone by the normal male.

Steroids having a 16α-hydroxyl group are not readily available or are difficult to synthesize. Hence this study was designed to obtain large amounts of urinary metabolites of 16α-hydroxydehydroisoandrosterone. The subject, a 24-year old normal male, was given 2.34×10^7 dpm in 465 μg of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone (Lot#1), by intravenous injection. At the same time 600 mg of 16α-hydroxydehydroisoandrosterone was administered orally, in 50 mg doses contained in gelatin capsules, over a period of twelve hours. After the injection of the labelled steroid urine was collected for five days, and each day's urine was extracted with ether. The steroid conjugates contained in the individual urines were hydrolysed first by solvolysis and then with β-glucuronidase. Table 14 shows the recovery of radioactivity in the

TABLE 14

Recovery of Radioactivity in the Sulfate and Glucosiduronidate Fractions of Urine

Day	Sulfates (dpm)	% Recovery of Injected Dose	Glucosiduronidates (dpm)	% Recovery of Injected Dose
1	2.95×10^6	12.6	1.28×10^7	54.8
2	1.51×10^5	0.65	2.43×10^5	1.04
3	1.69×10^4	0.07	3.60×10^4	0.15
4	--	--	1.24×10^4	0.05
5	--	--	5.00×10^3	0.002
Total	3.12×10^6	13.3	1.31×10^7	56.1

"sulfate" and "glucosiduronidate" fractions of each day's urine. There were insignificant amounts of radioactivity in the unconjugated fraction of the urine.

Since most of the recovered radioactivity was present in the urine of the first two days the extracts from this period were further processed. The sulfate fraction which weighed 406 mg and contained 3.10×10^6 dpm was chromatographed on a 100g silica gel column using increasing concentrations of ethanol in methylene chloride. The effluent from the column was collected at the rate of 30-40 ml/hour, and the peaks of radioactivity eluted are shown in Figure 3. The material within each peak was combined and the mode of elution, the weight and radioactivity of each residue are shown in Table 15. The residues of pools I and IV were not identified.

TABLE 15

Elution of Radioactive Material from the Initial Silica Gel Column

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	110-144	13.9	2.33×10^4
II	145-240	117.0	2.24×10^6
III	241-297	16.6	1.63×10^5
IV	298-411	30.1	1.07×10^5
V	412-464	31.1	3.14×10^5

The residue of Pool II was chromatographed on an 80 g Celite column using system A and three main radioactive peaks were eluted from the column as shown in Figure 4. Chromatography of the residue of IIA on two papers in system P for 18 hours gave one radioactive zone at an average distance of 19.5 cm. On elution a residue was obtained (9.1 mg and 3.62×10^5 dpm) which was further purified by chromatography on a 1 g silica gel column to yield 7.3 mg of

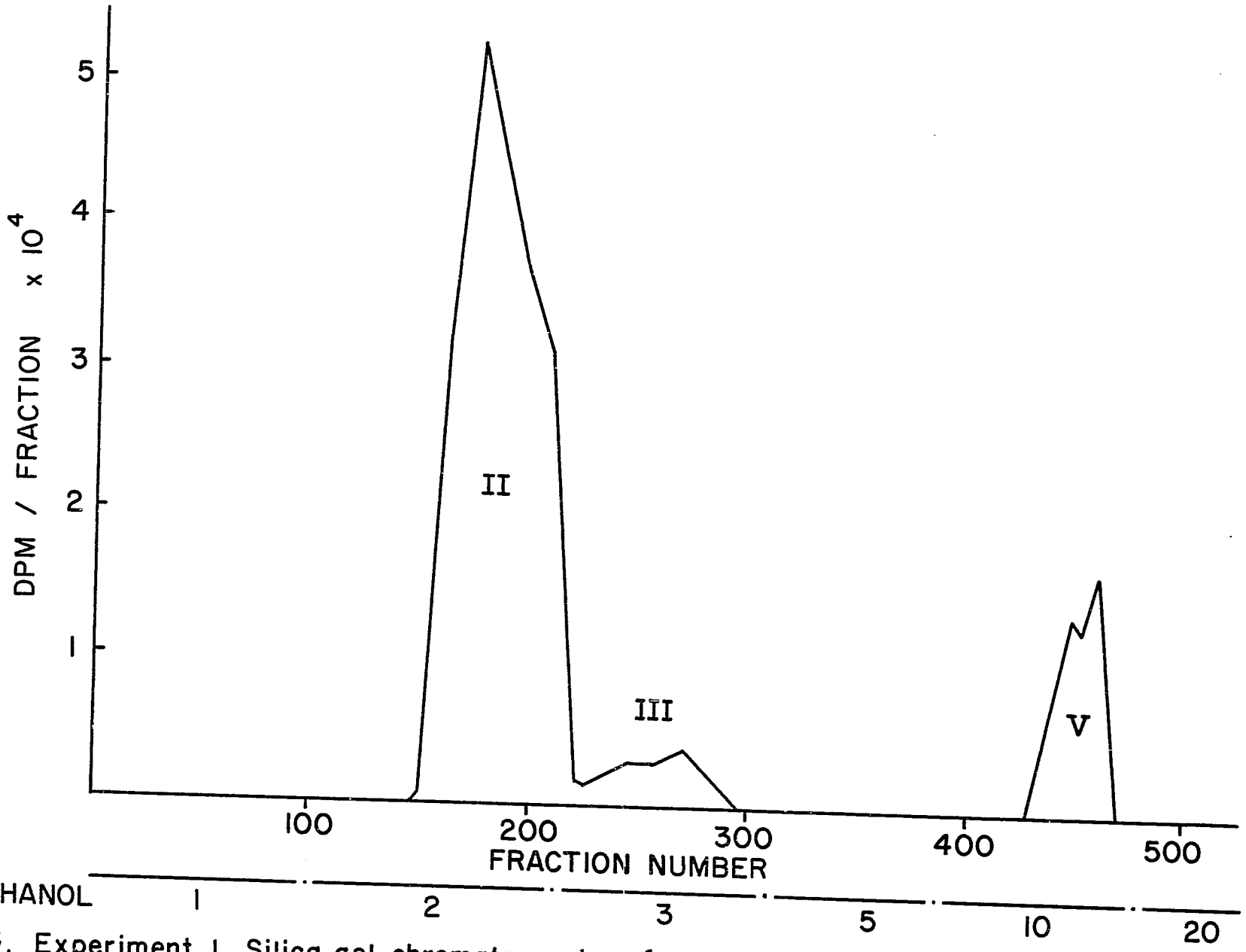

FIG.3. Experiment 1. Silica gel chromatography of extract containing sulfates.

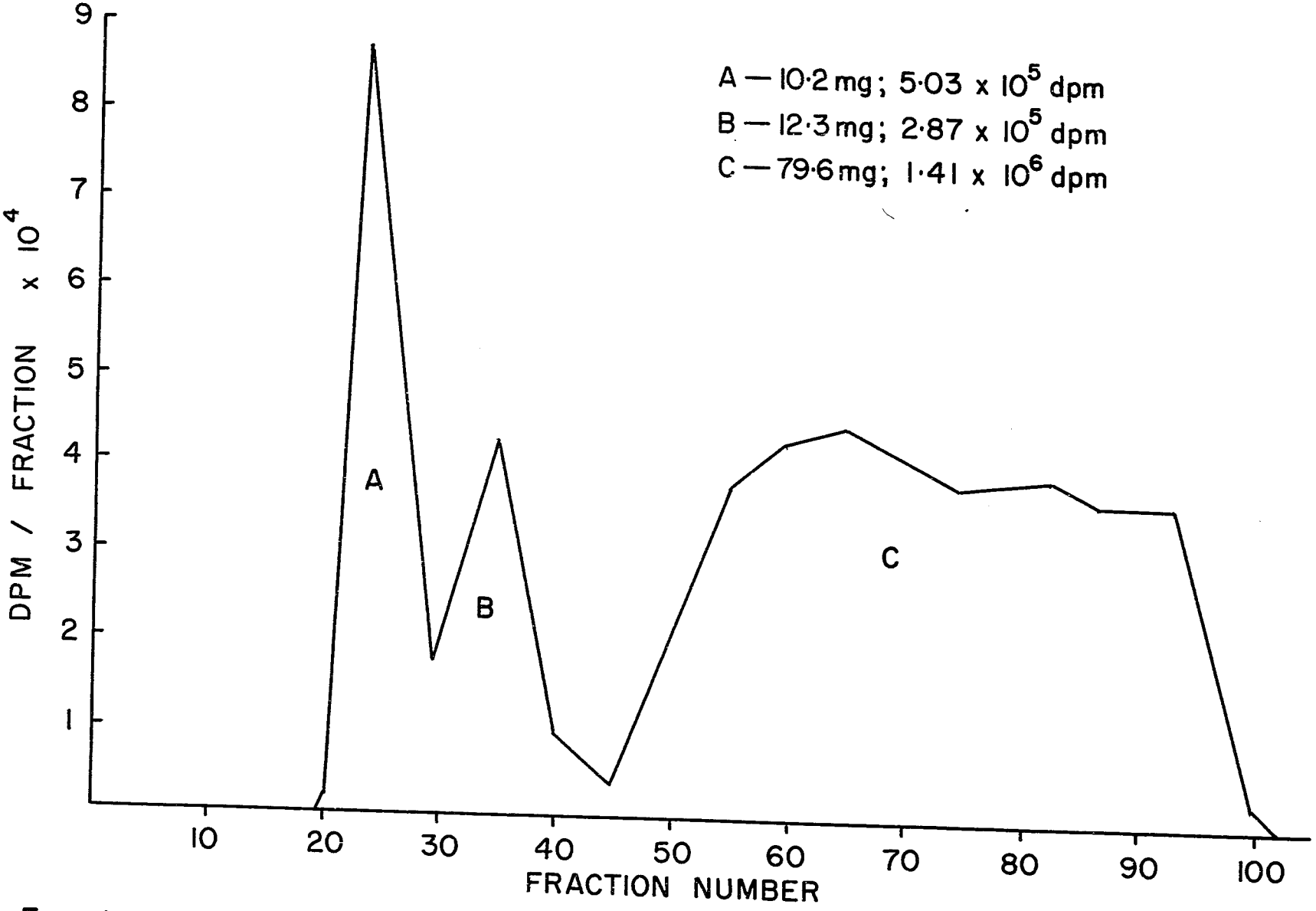

FIG. 4. Experiment I. Celite partition column. Sulfate fraction II. System A.

crystalline material containing 3.24×10^5 dpm. Crystallization of this material from acetone-Skellysolve B gave 2.9 mg of coarse needles, mp 181-183° C., specific activity 4.92×10^4 dpm/mg. An aliquot of the mother liquor (1.0 mg) was acetylated and the infrared spectrum (CS_2) of the product was identical to that of authentic 3α,16α-diacetoxy-5α-androstan-17-one. No attempt was made to crystallize the diacetate. Another aliquot of the mother liquor (2.9 mg) was reduced with $NaBH_4$ and the product was purified by chromatography on a 1 g silica gel column and crystallized from methanol-ether, yielding 0.9 mg of fine needles: mp 253-257°C, mmp 255-258°C; standard mp of androstanetriol 255-258°C; reported mp 254-256°C (32); specific activity 5.06×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of androstanetriol. It was not possible to obtain a sample of 16α-hydroxyandrosterone for direct comparison but the infrared spectrum (KBr) was identical to that of 16α-hydroxyandrosterone in the files of Dr. T.F. Gallagher, Institute for Steroid Research, Montefiore Hospital and Medical Center, New York.

Residue IIB, Figure 4, was chromatographed on two papers in system T. One radioactive peak was obtained with an average Rf of 0.63 and the eluate from the papers weighed 7.5 mg and contained 2.83×10^5 dpm. It was chromatographed on a 1 g silica gel column and crystallized with great difficulty from methanol-ethyl acetate. The crystals (3.3 mg) had a mp of 127-130°C, and the specific activity was 5.32×10^4 dpm/mg. When a solution of the crystals was applied on paper it gave a positive reaction with the B.T. reagent, a pink color when reacted with 77% sulfuric acid in absolute ethanol, a negative Zimmerman reaction and did not absorb UV light. The product obtained after reduction with $NaBH_4$ had the same infrared spectrum (KBr) as VC isolated from peak V, Figure 3, Table 15.

The residue from IIC, Figure 4, was chromatographed on a 70 g Celite column using system F and the rate of elution was 20 ml per hour (HBV=100 ml). One radioactive peak was obtained in the 4th-6th HBV. Fractions contained

within the peak were combined to yield 71.4 mg of material containing 1.14×10^6 dpm. It was crystallized from acetone-Skellysolve B to yield 40.1 mg of fine needles, mp 175-178°C, mmp 177-179°C; standard mp of 16α-hydroxydehydroisoandrosterone, 177-179°C , reported mp 177-181°C (19); specific activity 1.75×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of 16α-hydroxydehydroisoandrosterone.

Chromatography of the material from pool III, Figure 3, Table 15 on two papers in system T yielded one radioactive peak with an average R_f of 0.67 and the eluate from the papers was percolated through a 1 g silica gel column to yield 4.0 mg of yellow oil which contained 9.39×10^4 dpm. This oil could not be crystallized and some of it was accidentally lost. The remainder was acetylated and the product was percolated through a small alumina column to give 1.9 mg of colorless oil containing 3.32×10^4 dpm. This was crystallized from acetone-Skellysolve B yielding 1.1 mg of coarse plates: mp 188-190°C; standard mp of 3α,16α-diacetoxy-5β-androstan-17-one 185-188°C; specific activity 1.90×10^4 dpm/mg. Its infrared spectrum (CS_2) was identical to that of 3α,16α-diacetoxy-5β-androstan-17-one. The calculated specific activity of the 16α-hydroxyetiocholanolone was 2.49×10^4 dpm/mg.

The residue of pool V, Figure 3, Table 15, was crystallized directly from methanol to give 4.7 mg of fine needles; mp 258-262°C. Its infrared spectrum (KBr) was identical to that of androst-5-ene-3β,16α,17β-triol (Hirschmann's triol). The crystals were chromatographed on thin layer plates in system benzene-methanol (75:25) and the material eluted from the plates was crystallized from methanol to yield 4.5 mg of fine needles: mp 266-270°C; mmp 265-267°C; standard mp of Hirschmann's triol 264-266°C; reported mp 265-270°C (31); specific activity 1.80×10^4 dpm/mg.

After crystallization of the residue from pool V, the mother liquor

was chromatographed on one paper in system O for 37 hours and it resolved into three radioactive peaks VA, VB and VC, which had mobilities of 13.4 cm, 21.3 cm, and 31.2 cm respectively. The eluate of VA which weighed 3.7 mg and contained 5.08×10^4 dpm was percolated through a 1 g silica gel column and the material eluted was crystallized from methanol to yield 0.8 mg of fine needles which were identical to Hirschmann's triol present in the crystals.

From VB an eluate was obtained which weighed 3.3 mg and contained 8.99×10^4 dpm. Chromatography on a 1 g silica gel column and elution with 5 % ethanol in methylene chloride gave 2.2 mg of crystalline material containing 7.97×10^4 dpm. Crystallization from methanol afforded 1.0 mg of small plates: mp 255-257°C; mmp with androstanetriol 253-256°C; specific activity 5.05×10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of androstanetriol.

Further purification of the eluate from VC (3.6 mg and 6.08×10^4 dpm) was achieved by percolating it through a 1 g silica gel column and the material eluted was crystallized from methanol to yield 0.7 mg of fine cubes: mp 270-273°C, specific activity 4.99×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to the $NaBH_4$ reduced product of the unknown IIB, but did not correspond to any known C-19 triol. At this stage it was evident that the $NaBH_4$ reduction product of IIB and the material in VC were identical.

The glucosiduronidate fraction from the first two days' urine weighed 570 mg and contained 1.41×10^7 dpm. It was chromatographed on a 150 g silica gel column as previously described for the sulfate fraction. Aliquots from every fourth fraction were taken for counting and the various radioactive peaks obtained are shown in Figure 5. The origin, weight, and radioactivity of each residue are shown in Table 16.

Pool I (29.1 mg and 5.42×10^5 dpm) was chromatographed on four papers

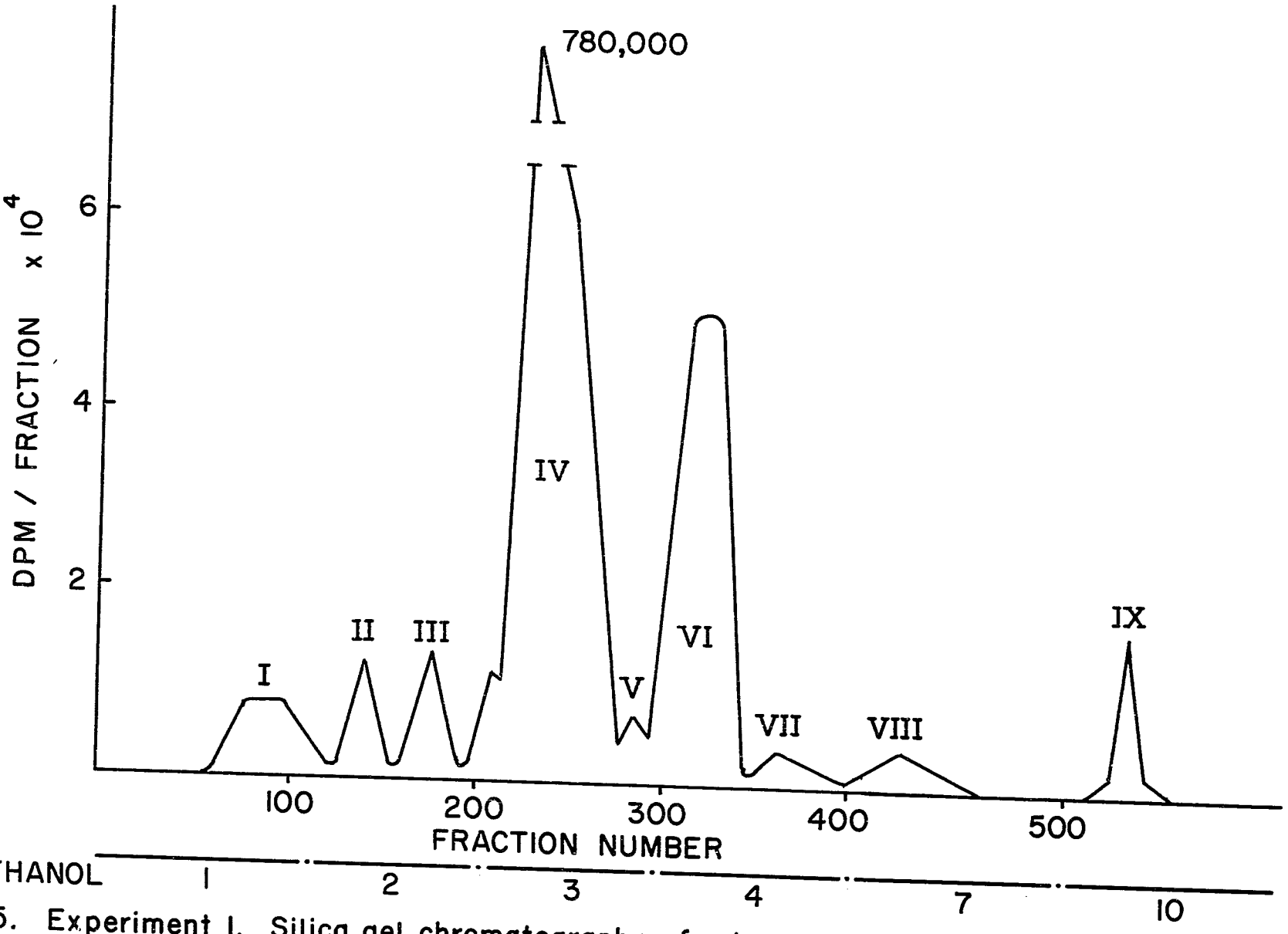

FIG. 5. Experiment I. Silica gel chromatography of glucosiduronidate fraction.

TABLE 16

Elution of Radioactive Material from the Initial Silica Gel Column

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	65-136	29.0	5.42×10^5
II	137-163	18.0	1.48×10^5
III	164-200	12.9	2.30×10^5
IV	201-265	230.1	1.11×10^7
V	266-288	14.4	2.35×10^5
VI	289-340	63.6	1.30×10^6
VII	341-380	14.1	1.84×10^5
VIII	381-484	46.5	2.82×10^5
IX	485-544	38.7	3.18×10^5

in system W for 24 hours. One radioactive peak was obtained at an average distance of 26.3 cm. The material eluted weighed 8.1 mg and contained 2.67×10^5 dpm. It was percolated through a small silica gel column and elution with 20% ether in benzene gave 6.0 mg of crystals containing 2.45×10^5 dpm. Crystallization from acetone:n-hexane gave 4.4 mg of small plates; mp 195-196°C, specific activity 4.96×10^4 dpm/mg. The infrared spectrum (KBr) is shown in Figure 6. This unknown was Zimmerman negative, U.V. negative, gave a slight color with 77% sulfuric acid in absolute ethanol and a positive Blue Tetrazolium reaction after 5 minutes. The mass spectrum performed by Dr. C. Djerassi, Stanford University, California, indicated that the unknown had 42 mass units more than 16α-hydroxydehydroisoandrosterone. A high-resolution mass spectrum analysis, Table 17, (courtesy of Dr. C.

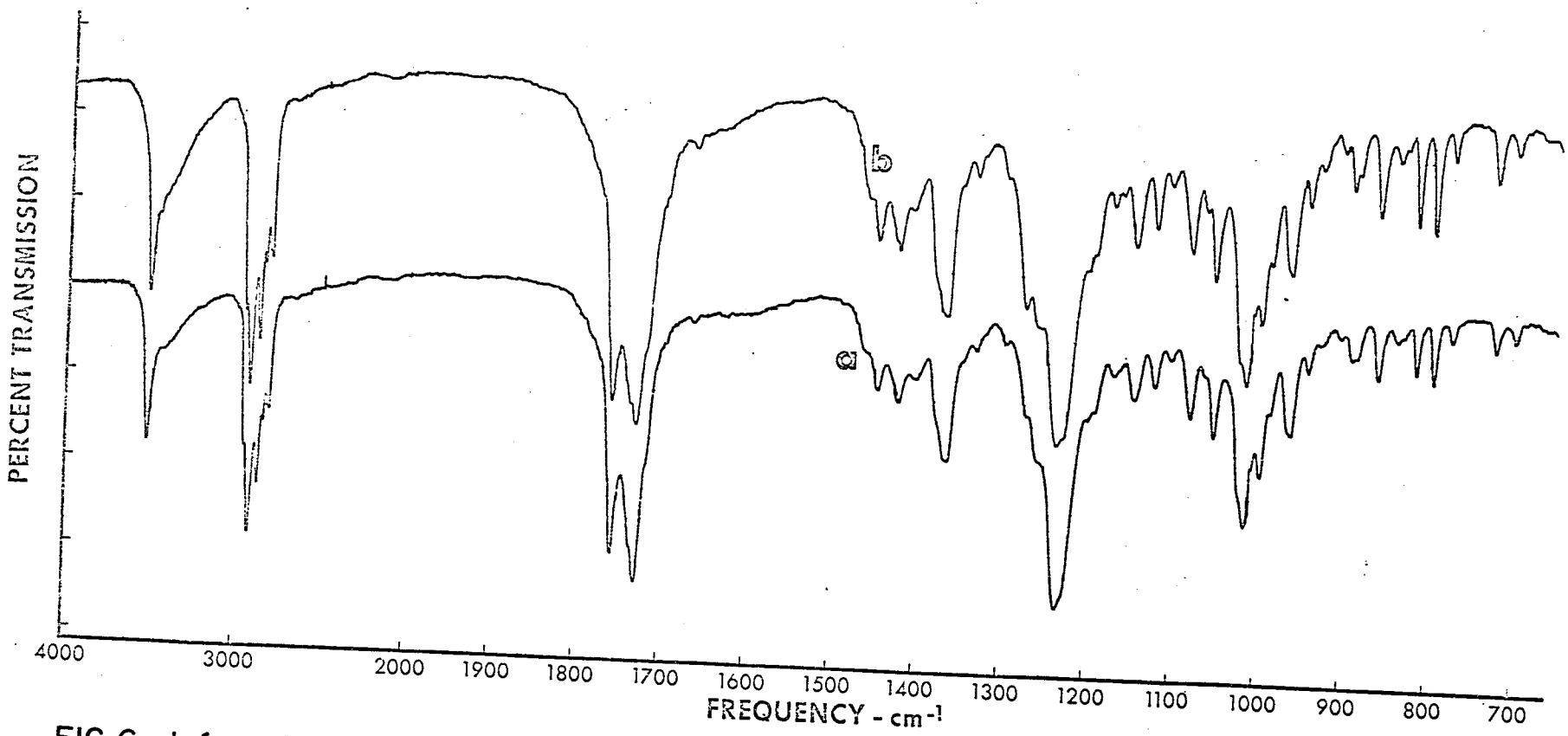

FIG.6. Infrared spectra (KBr). (a) Metabolite isolated from pool I of glucosiduronidate fraction of urine. (b) Product synthesized from 3α,16α-dihydroxyandrost.-5-en-17-one.

TABLE 17

Mass Spectrum Analysis of Crystalline Metabolite from Pool 1

Nominal m/e	Accurate m/e	Calculated m/e	Formula
346	346.21466	346.21440	$C_{21}H_{30}O_4$
286	286.15674	286.15689	$C_{18}H_{22}O_3$
	286.19290	286.19327	$C_{19}H_{26}O_2$

Djerassi) of this unknown indicated that it had a molecular weight of 346 which would correspond to the monoacetate of a steroid such as 16α-hydroxy-dehydroisoandrosterone. This is strengthened by a strong absorption band at 123! cm^{-1} in the infrared and by the existence of a peak in the mass spectrum at m/e 286 which corresponds to the loss of acetic acid. The 100 Mc NMR spectrum (courtesy of Dr. C. Djerassi) showed peaks at 4.08 ppm, 5.5 ppm and 5.57 ppm. In the NMR spectrum of 3α,16α-diacetoxyandrost-5-en-17-one the 5.57 ppm peak remained the same but there was an upfield shift of the 3β-proton from 4.08 ppm to 5.03 ppm and the 16β proton from 4.42 ppm to 5.5 ppm when compared to the spectrum of the unacetylated compound (Figure 8a,b page65). An examination of the infrared spectrum of the unknown showed carbonyl absorption at 1758 cm^{-1} which is consistent with the presence of a 16α-acetoxy-17-ketone group in ring D. All these results together permit the assignment of the structure 16α-acetoxy,3α-hydroxyandrost-5-en-17-one to the metabolite in pool I. Since this unknown had the same infrared spectrum (KBr) as that of an acetate IVB2 (page 66), mp 194-195°C, mmp 192-193°C, the structure of the latter was also established.

The residue from pool III, Table 16, was chromatographed on two papers in system N for 6 hours and one radioactive area was observed with the same mobility as 16α-hydroxyandrostenedione. The eluate from the papers weighed 10.0 mg and contained 1.36×10^5 dpm. Chromatography of this eluate on a

small silica gel column and elution with 60% ether in benzene yielded 2.0 mg of yellow oil which contained 8.80×10^4 dpm. The infrared spectrum (KBr) of an aliquot of this material was similar to that of 16α-hydroxyandorstenedione. The material present in the KBr disc was recovered and mixed with the original material. Chromatography of the mixture in system Q for 4 hours and purification of the eluate on a small silica gel column afforded 0.2 mg of a residue containing 1.80×10^4 dpm. This was then mixed with carrier 16α-hydroxyandrostenedione and crystallized to constant specific activity as shown in Table 18.

TABLE 18

Proof of Radiochemical Purity of 16α-Hydroxyandrostenedione Isolated from the Glucosiduronidate Fraction of Normal Male Urine

Crystallization	Specific Activity (dpm ^{3}H/mg)			
	16α-Hydroxyandrostenedione		16α-Acetoxyandrostenedione	
	Crystals	Mother Liquor	Crystals	Mother Liquor
1	980	4260	840	860
2	990	1010		
3	970	1000		
Calculated*	1200		870	

*A total of 1.80×10^4 dpm was mixed with 14.9 mg carrier. The third crystals and mother liquor were used to prepare the acetate. There was insufficient material for further crystallization.

Pool IV, Table 16, Figure 5, yielded a residue which was chromatographed on a 200 g Celite column using system A. Aliquots from every fourth fraction were taken for counting. Three main peaks of radioactivity were obtained as shown in Figure 7. The material in fractions 50-80, 81-126 and 127-156 were combined and labelled IVA, IVB and IVC, respectively. The residue from IVA (1.95×10^6 dpm and 46.0 mg) was rechromatographed on a 47 g Celite column in system A (HBV-60 ml). Aliquots from every second fraction were

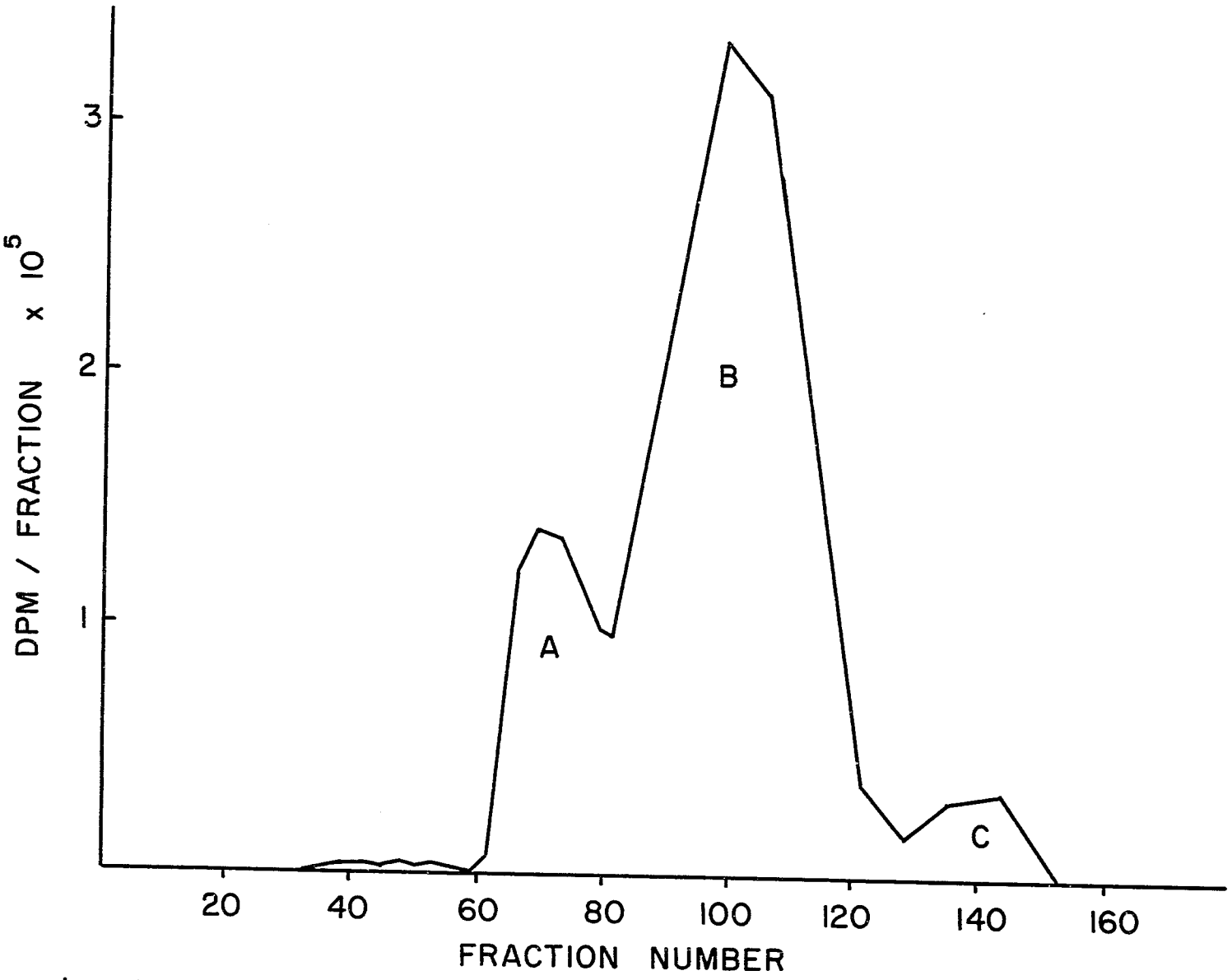

FIG. 7. Experiment I. Celite column chromatography. Glucosiduronidate fraction IV. System A.

taken for counting. One major peak of radioactivity was obtained in the 3rd and 4th HBV and it contained 43.0 mg of crystalline material which had 1.82×10^6 dpm. Crystallization from acetone yielded 26.9 mg of coarse needles: mp 185-189°C, specific activity 4.79×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of 16α-hydroxyandrosterone isolated from the sulfate fraction.

From pool IVB, Figure 7, a residue was obtained which weighed 150 mg and contained 7.65×10^6 dpm. It was chromatographed on a 140 g Celite column in system F (HBV-180 ml). Measurement of radioactivity from every third tube indicated the presence of one symmetrical peak of radioactivity in the 4th HBV. The material within this peak was pooled to give 129 mg of amorphous residue which contained 6.93×10^6 dpm. It was crystallized with great difficulty from n-hexane:ether:acetone to yield 56.0 mg of slightly yellow fine plates: mp 125-129°C, specific activity 5.29×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of the unknown IIB from the sulfate fraction and gave the same color reactions previously described for that unknown. An aliquot of IVB containing 10.8 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 30-40% benzene in Skellysolve B gave 8.1 mg of colorless oil, IVBI, and with 20% ether in benzene 3.2 mg of an oily residue was found, IVB2. The oil IVBI was crystallized from n-hexane to yield 2.8 mg of coarse plates, mp 156-159°C. The specific activities of the crystals and mother liquor were 1.14×10^4 and 1.12×10^4 dpm ^{14}C/mg ($^3H/^{14}C = 2.2$), respectively, indicating the presence of two acylable hydroxyl groups. At this point 10 mg of the unacetylated compound, IVB and 10 mg of the diacetate were sent to Dr. J. Fishman at the

Montefiore Hospital and Medical Center, N.Y., for nuclear magnetic resonance analysis. The spectra obtained are shown in Figure 8 (a and b). The top scan in each figure represents a computer spectrum at 4/3 scale of the bottom one as far as interpeak distances are concerned. A 3β proton absorption was observed at 4.08 ppm in the unacetylated compound and 5.03 ppm in the diacetate indicating the presence of an equatorial 3β proton. When compared to the spectrum of 16α-hydroxydehydroisoandrosterone there was no change in the C-6 proton, 16β proton and C-18 and C-19 methyl group absorptions. The mass spectrum (courtesy of Dr. C. Djerassi) indicated that the compound had a molecular weight of 304, consistent with the structural formula $C_{19}H_{28}O_3$. These data indicated that the unknowns IIB from the sulfate fraction and IVB from the glucosiduronidate fraction were 3α,16α-dihydroxy-androst-5-en-17-one. The reduced unknown VC from the sulfate fraction could therefore be assigned the structure of androst-5-ene-3α,16α,17β-triol.

Confirmatory evidence for the structure of IVB was furnished by the platinum-catalysed hydrogenation of the diacetate which was first reduced with $NaBH_4$. Ten mg of IVB was acetylated and the diacetate was chromatographed on an alumina column to yield 11.2 mg of an oily residue. Reduction with $NaBH_4$ and chromatography of the product on a small alumina column yielded 9.8 mg of colorless oil. To a solution of this oil in 10 ml of ethyl acetate was added 2 drops of 70% perchloric acid and the mixture was shaken in a hydrogen atmosphere with 60 mg of platinum oxide under slight pressure for 20 hours. The product formed was chromatographed on an alumina column to yield 6.7 mg of yellow oil. This oil was saponified with 0.4M methanolic KOH and the product was percolated through a small silica gel column. Elution with 5% ethanol in methylene chloride gave 4.2 mg of crystalline material. Recrystallization from methanol afforded 1.9 mg of coarse granules: mp 274-276^{o}C,

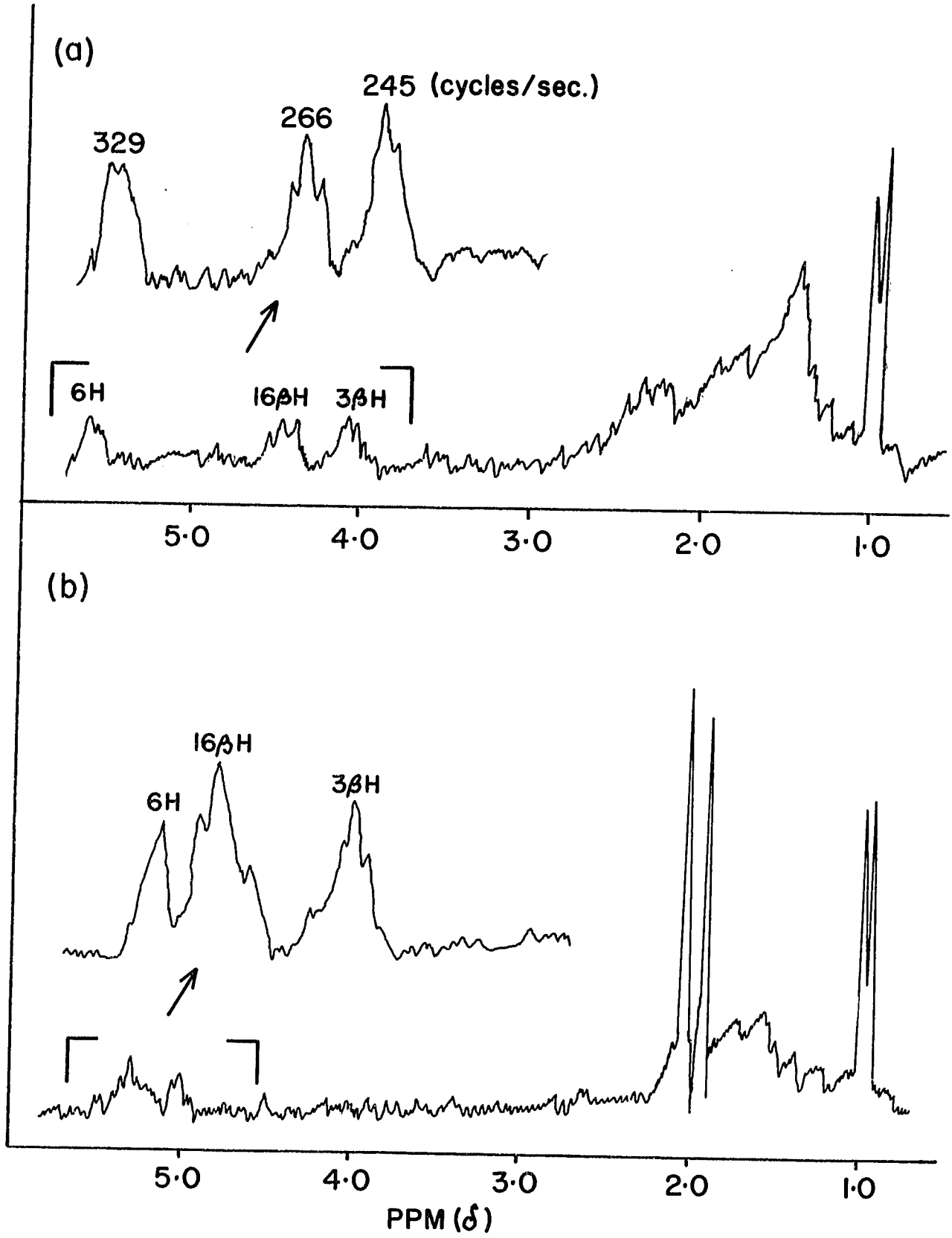

FIG. 8. N.M.R. Spectra. Internal standard — Trimethylsilane.
(a) Unknown I V B. (b) Diacetate of I.V B.
The enlarged top scans represent computer spectra at 4/3 scale of the bottom ones as far as interpeak distances are concerned.

mmp 275-277°C, standard etiocholanetriol mp 275-278°C; reported mp 276-278°C (32). The infrared spectrum (KBr) was identical to that of etiocholanetriol. This established the presence of a 3α-hydroxy-5-ene moiety since it has been shown by Lewis and Shoppee (170) and Fukushima et al (37) that a 3α-acetoxy-5-ene group on catalytic hydrogenation gives mainly the 5β-reduced product (confirmed in our laboratory with the model steroid, 3α-acetoxyandrost-5-en-17-one).

The residue of IVB2 obtained after acetylation with labelled acetic anhydride was crystallized from acetone-Skellysolve B to yield 2.0 mg of coarse plates, mp 192-193°C, specific activity 5.07×10^3 dpm ^{14}C/mg ($^3H/^{14}C$ = 4.4), indicating that by comparison with IVB1 a single hydroxyl group in IVB was acetylated. This compound gave a positive Blue Tetrazolium reaction after 5 minutes, a slight color with 77% sulfuric acid in ethanol and negative results with Zimmerman reagent, UV absorption, and had the identical infrared spectrum (KBr) as 16α-acetoxy,3α-hydroxyandrost-5-en-17-one.

Pool IVC, Figure 7, yielded a residue which contained 5.07×10^5 dpm and weighed 8.0 mg and it was chromatographed on two thin layer plates in system n-hexane:ethyl acetate (1:2). The area corresponding to 16α-hydroxydehydroisoandrosterone was eluted to give 5.5 mg of crystalline material containing 3.75×10^5 dpm. Crystallization of this material from acetone:Skellysolve B yielded 2.0 mg of fine needles: mp 177-180°C, nmp with 16α-hydroxydehydroisoandrosterone 177-179°C, specific activity 8.36×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of 16α-hydroxydehydroisoandrosterone.

The residue of pool V, Figure 5, Table 16, was chromatographed on a 60 g. Celite column using system D, and the distribution of radioactivity is shown in Figure 9. Fractions 19-31 were pooled to give 53.4 mg of yellow oil containing 1.18×10^6 dpm. This material was chromatographed on eight

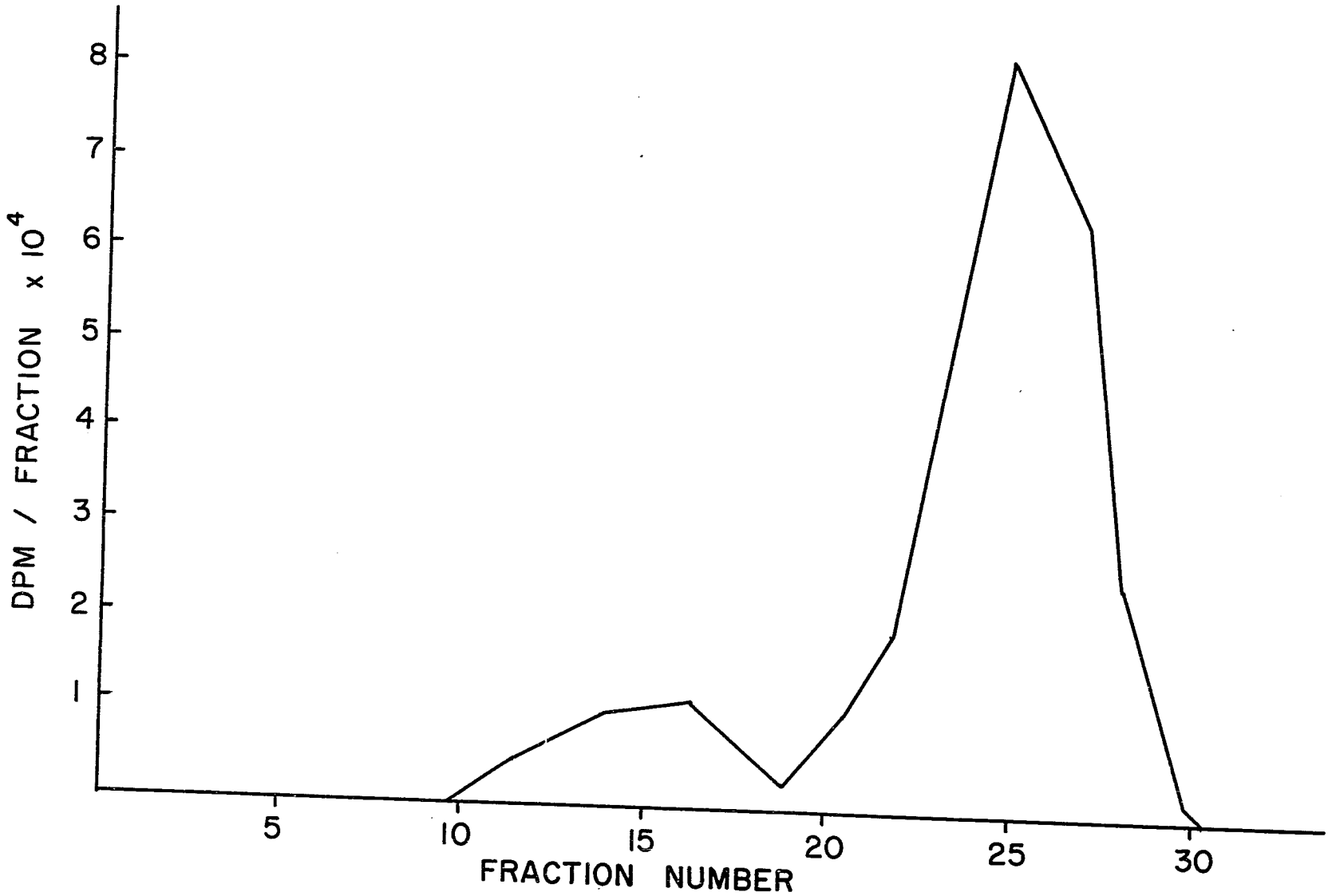

FIG. 9. Experiment I. Celite column chromatography. Glucosiduronidate fraction V. System D.

papers in system Q for five hours. A single radioactive band with an average mobility of 16.1 cm was observed, which on elution gave 61.0 mg of a white residue containing 8.75×10^5 dpm. Chromatography on a 6 g silica gel column and elution with 2% and 3% ethanol in methylene chloride afforded 30.3 mg of colorless oil containing 7.45×10^5 dpm. An aliquot containing 6.22×10^4 dpm was acetylated and after purification on a small alumina column the acetate was crystallized from acetone-heptane to give 0.8 mg of small plates: mp 181-185°C, specific activity 1.77×10^4 dpm/mg; calculated specific activity of the unacetylated compound 2.26×10^4 dpm/mg. Its infrared spectrum (CS_2) was identical to that of 3α,16α-diacetoxy-5β-androstan-17-one. The remainder of the colorless oil was crystallized from acetone-Skellysolve B to yield 15.8 mg of fine crystals: mp 157-160°C, specific activity 2.44×10^4 dpm/mg. It was not possible to obtain a sample of the unacetylated compound for direct comparison.

From pool IX, Figure 5, Table 16, a residue was obtained which was chromatographed on a 44 g Celite column using system L, and the distribution of radioactivity obtained is shown in Figure 10. The material in fractions 29-48 were pooled to give 11.1 mg of residue containing 2.09×10^5 dpm. Crystallization from methanol afforded 2.3 mg of fine needles: mp 265-269°C, specific activity 5.26×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of androst-5-ene-3α,16α,17β-triol isolated from the sulfate fraction. The residues of pools II, VI, VII and VIII were not identified.

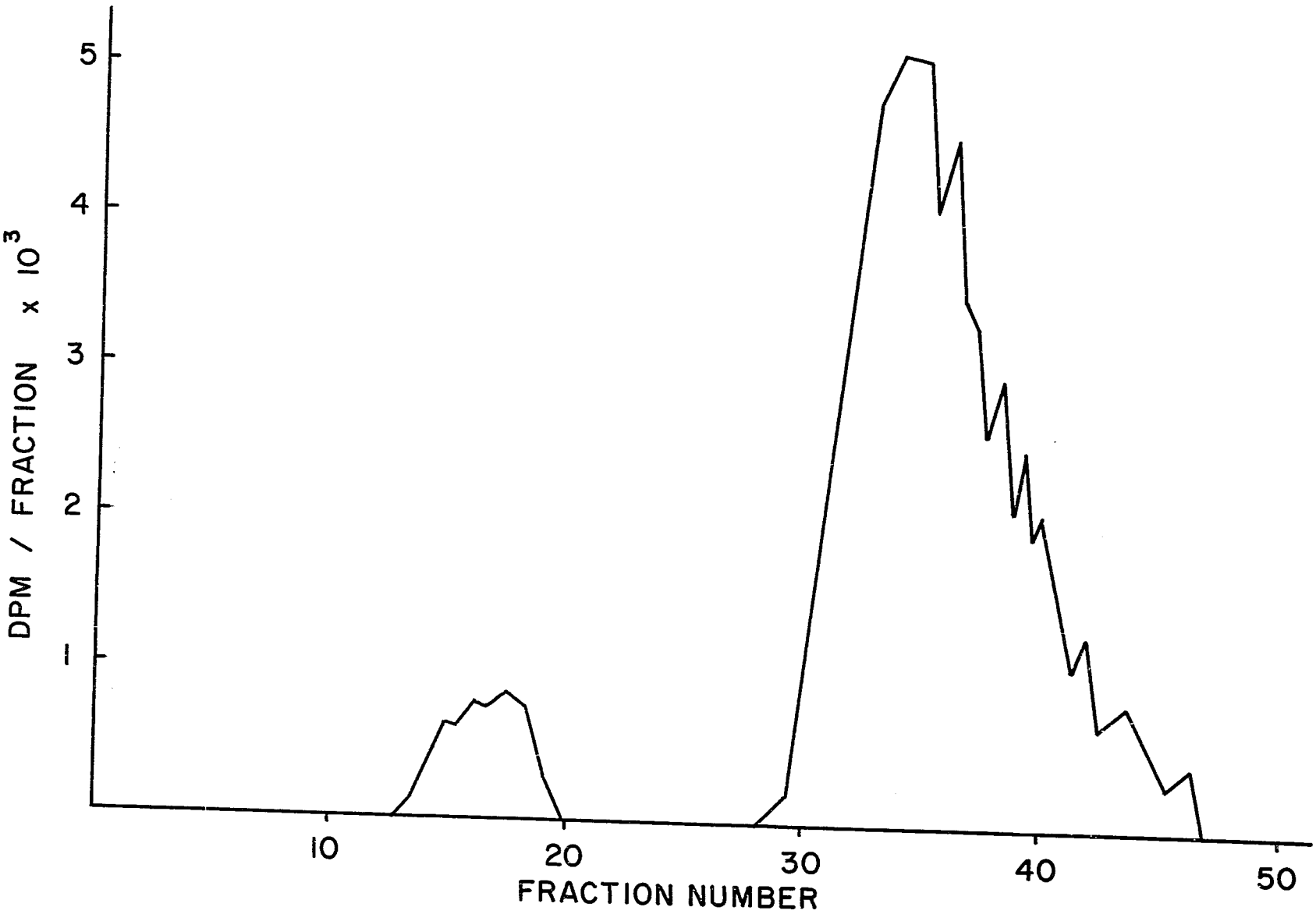

FIG. 10. Experiment I. Celite column chromatography. Glucosiduronidate fraction IX. System L.

EXPERIMENT 2. Metabolism of 16α-Hydroxydehydroisoandrosterone by the Pregnant Female.

A normal 23-year old subject in the 36th week of pregnancy was injected with 1.50×10^7 dpm of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone (Lot # 1) in 310 µg. Urine was collected for five days and each day's urine was extracted with ether, solvolysed and then hydrolysed with β-glucuronidase. The total radioactivity recovered in each day's urine and the amount present as sulfates and glucosiduronidates are shown in Table 19. There were insignificant amounts of radioactivity in the unconjugated fraction of the urines. Since most of the radioactivity was present in the first two days the extracts from this period were further processed.

The sulfate fraction weighed 660 mg and contained 1.58×10^6 dpm. It was chromatographed on a 170 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 11. The mode of elution of the radioactive material and the contents of each pool are shown in Table 2

TABLE 20

Residues 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	132-240	45.3	2.24×10^5
II	241-340	31.8	1.75×10^5
III	341-526	56.3	1.59×10^5
IV	527-600	36.1	1.50×10^5
V	601-686	25.3	2.28×10^5

Pool II, Figure 11, Table 20, gave a residue which was chromatographed on paper in system Q for 8 hours. Two radioactive zones, IIA and IIB, were

TABLE 19

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	Glucosiduronidates	% recovery of injected dose
1	1.16×10^7	77.1	1.39×10^6	9.0	9.93×10^6	64.5
2	7.45×10^5	4.95	1.90×10^5	1.2	4.76×10^5	3.1
3	1.48×10^5	0.98	1.40×10^4	0.1	8.30×10^4	0.5
4	9.86×10^4	0.66	--	-	4.00×10^4	0.2
5	3.97×10^4	0.26	--	-	2.32×10^4	0.1
Total	1.26×10^7	83.95	1.59×10^6	10.3	1.06×10^7	68.4

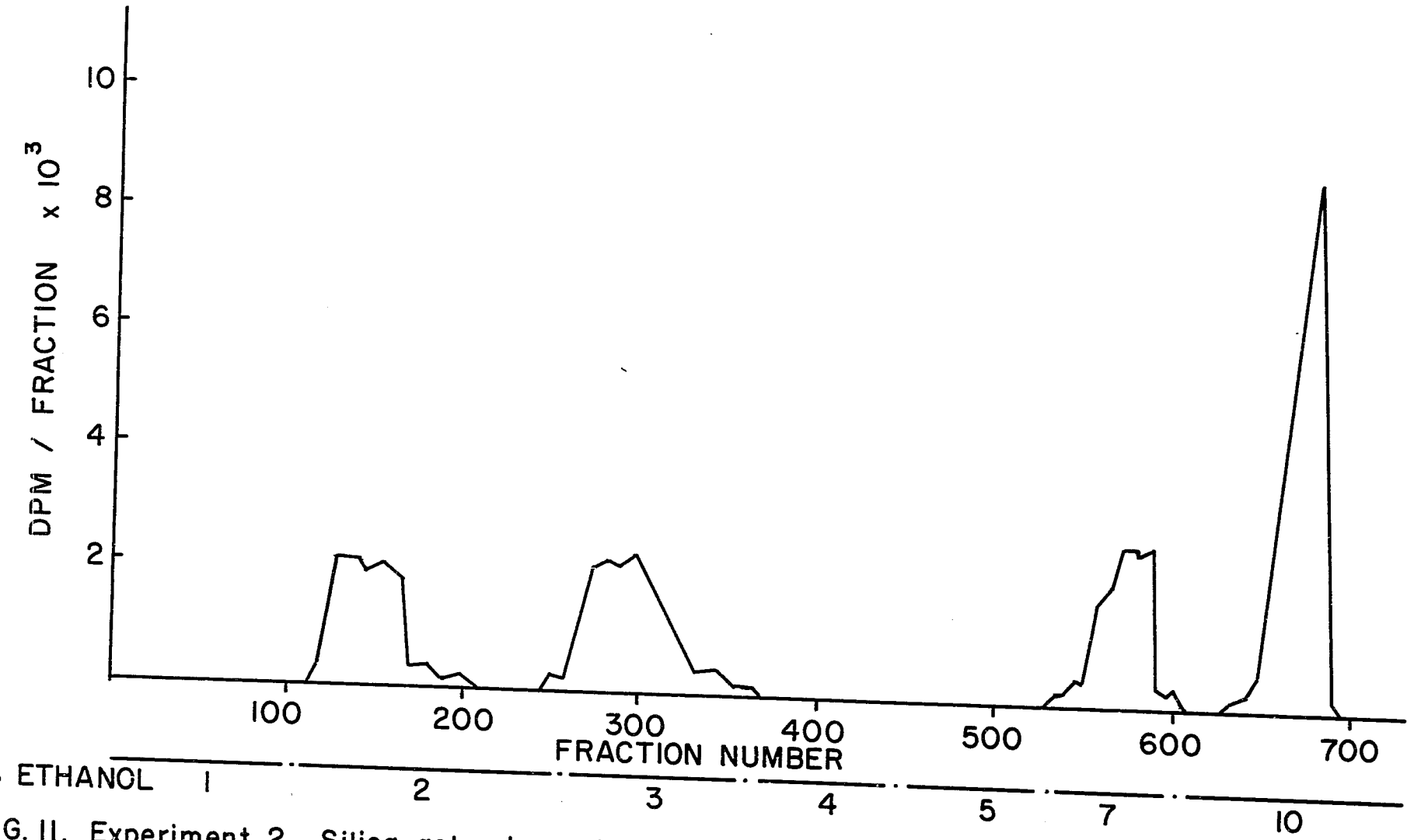

FIG. 11. Experiment 2. Silica gel chromatography. Sulfate fraction.

obtained with mobilities of 15.7 cm and 30.1 cm, respectively. Zone IIA corresponded in mobility to 16α-hydroxydehydroisoandrosterone and when eluted weighed 12.2 mg and contained 3.92×10^4 dpm. Chromatography on a 1 g silica gel column afforded 2.44×10^4 dpm in 1.7 mg of yellow oil which could not be crystallized. Accordingly, it was acetylated with acetic anhydride, solution No. 2, and then mixed with 20.3 mg of carrier 3β,16α-diacetoxyandrost-5-en-17-one. The mixture was percolated through a small alumina column to yield 2.12×10^4 dpm of ^3H and 20.0 mg of crystalline material. This material was crystallized to constant specific activity and again after $NaBH_4$ reduction as shown in Table 21. From the $^3H/^{14}C$ ratio, the specific activity of the 16α-hydroxydehydroisoandrosterone was calculated to be 4.11x105 dpm/mg.

Zone IIB corresponded in mobility to 16α-hydroxyandrosterone and after elution the residue weighed 8.8 mg and contained 4.57×10^4 dpm. It was chromatographed on a 1 g silica gel column to yield 0.9 mg of yellow oil having 3.41×10^4 dpm. This oil was acetylated with ^{14}C-acetic anhydride solution No. 2 and the product formed was chromatographed on a 1 g alumina column. One radioactive peak was eluted from the column having an average $^3H/^{14}C$ ratio of 16.8. Chromatography on paper in system W gave a single peak with an R_f of 0.9 and $^3H/^{14}C$ ratio of 23.6. Elution from the paper and rechromatography on AC81 paper using reversed phase in system W gave one radioactive peak with an R_f of 0.67 and $^3H/^{14}C$ ratio of 17.3. At this stage there was insufficient material for further chromatography and the specific activity of the metabolite was 2.53×10^5 dpm/mg.

The residue of pool IV, Figure 11, Table 20, was chromatographed on three papers in system T for 14 hours. One radioactive band was observed with the same mobility as estriol run alongside. On elution it gave 4.15 $\times 10^4$ dpm and 11.9 mg of brown oil which was purified by chromatography on a 1 g silica gel column to yield 4.2 mg of material having 3.46×10^4 dpm.

TABLE 21

Proof of Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated from the Sulfate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm/mg)							
	3β,16α-Diacetoxyandrost-5-en-17-one				3β,16α-Diacetoxyandrost-5-en-17β-ol*			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	1270	2.8	710	2.9	1260	2.9	1160	2.9
2	1280	2.8	1300	2.8	1200	2.9	1210	2.9
3	1280	2.9	1270	2.9				
Calculated**	1050							

*The third crystals and mother liquor of the diacetate were reduced with $NaBH_4$ and the product was chromatographed on a small alumina column prior to crystallization.

**After chromatography of acetylated IIA with carrier 3β,16α-diacetoxyandrost-5-en-17-one on a small alumina column, 20.0 mg of crystalline material was obtained containing 2.10×10^4 dpm ^3H. The calculated specific activity is based on these values.

Crystallization from methanol afforded 0.9 mg of fine needles: mp 274-276°C, standard mp of estriol 276-279°C, reported mp 280°C (171), specific activity 2.70×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of estriol. To the rest of the crystals were added 19.6 mg of carrier estriol and the mixture crystallized to constant specific activity and again after the formation of the triacetate as shown in Table 22.

TABLE 22

Radiochemical Purity of Estriol Isolated from Pool IV of the Sulfate Fraction of Pregnancy Urine

	Specific Activity (dpm ^3H/mg)			
Crystal-	Estriol		Estriol triacetate*	
lization	Crystals	Mother Liquor	Crystals	Mother Liquor
1	410	380	290	290
2	420	400	290	280
Calculated	-		290	

*The second crystals and mother liquor were used in the formation of this derivative. The calculated specific activity of the triacetate was based on the final specific activities of estriol and altered molecular weight.

Pool V, Figure 11, Table 20, was chromatographed on five papers in system L for four hours. One radioactive zone was obtained having the same mobility as androst-5-ene-3β,16α,17β-triol. On elution 42.4 mg of a white residue was obtained having 1.40×10^5 dpm. This residue was chromatographed on four thin layer plates using benzene:methanol (3:1). The eluate from the plates weighed 13.3 mg and contained 1.35×10^5 dpm. Crystallization from methanol afforded 1.0 mg of fine needles: mp 262-266°C, mmp with androst-

5-ene-3β,16α,17β-triol 261-266°C, specific activity 6.77×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of Hirschmann's triol. A total of 4.31×10^4 dpm of the crystals was mixed with carrier and crystallized as shown in Table 23. The residues in pools I and III were not processed.

TABLE 23

Proof of Radiochemical Purity of Hirschmann's Triol Isolated from Pool V of Pregnancy Urine

Crystallization	Specific Activity (dpm/mg) Crystals	Mother Liquor
1	2070	2100
2	2090	2120
Calculated*	2160	

*A total of 4.31×10^4 dpm of crystals from pool V was mixed with 20 mg of carrier Hirschmann's triol.

The glucosiduronidate fraction (600 mg and 1.04×10^7 dpm) was chromatographed on a 150 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. In Figure 12 is shown the distribution of radioactivity from which the different pools shown in Table 24 are obtained. Pools I, II, V, VII, VIII and X contained material which could not be identified.

The residue of Pool III, Table 24, Figure 12, was chromatographed on three papers in system N for 29 hours. One radioactive peak was obtained having the same mobility as 16α-hydroxyandrostenedione. On elution it gave 17.2 mg containing 5.58×10^5 dpm. Chromatography on three papers in system W for 24 hours afforded 4.9 mg of colorless oil having 3.94×10^5 dpm. This material was rechromatographed on one paper in system Q for 4 hours and one symmetrical zone

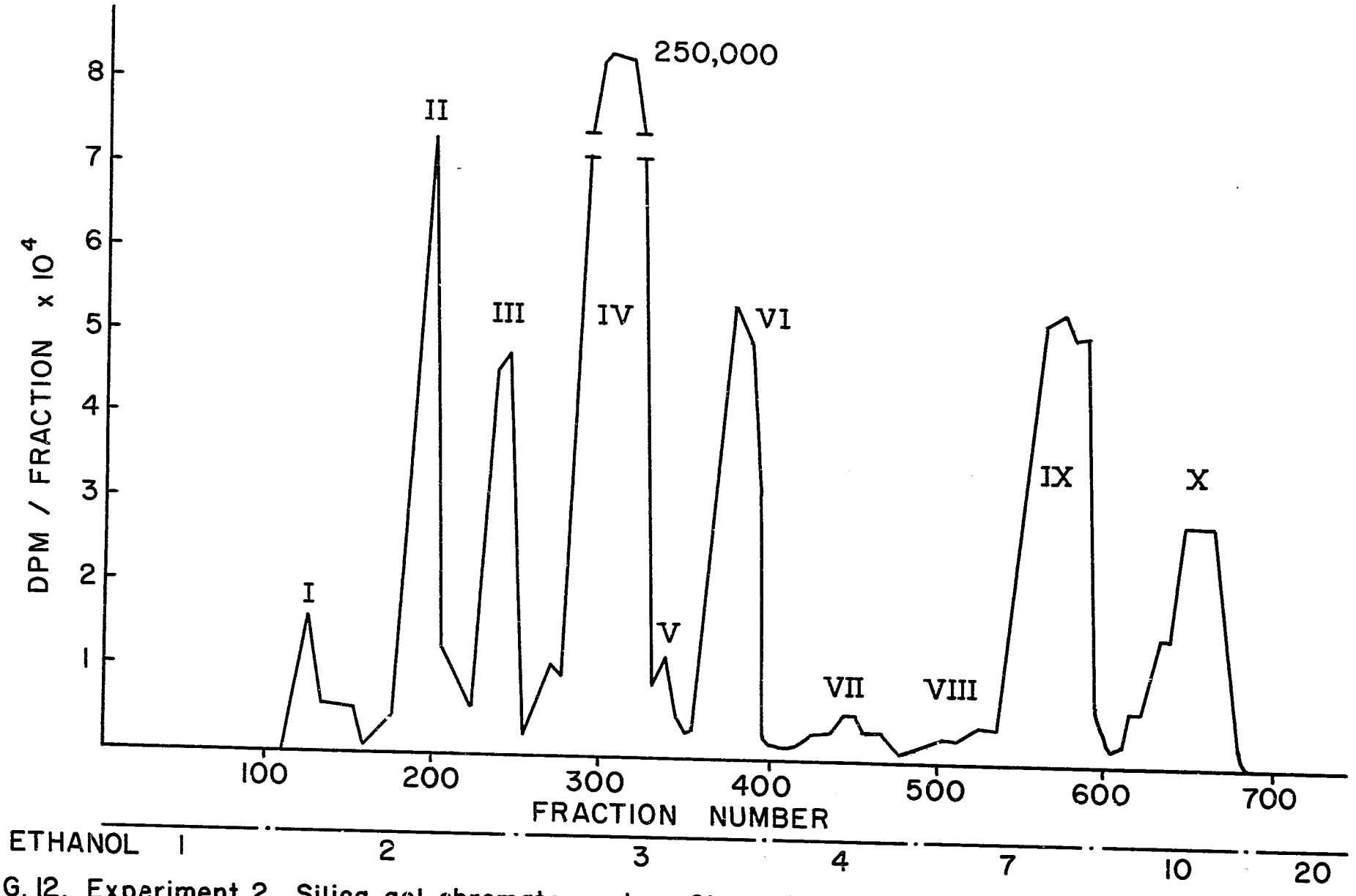

FIG. 12. Experiment 2. Silica gel chromatography. Glucosiduronidate fraction.

TABLE 24

Residues from the Initial Silica Gel Column after Chromatography of the Extract from the Glucosiduronidate Fraction of Pregnancy Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	119-160	63.4	2.54×10^5
II	161-222	29.5	9.30×10^5
III	223-250	23.6	6.32×10^5
IV	251-320	98.7	3.34×10^6
V	321-344	13.5	2.23×10^5
VI	345-388	18.7	9.42×10^5
VII	389-400	53.4	3.01×10^5
VIII	401-520	39.5	1.58×10^5
IX	521-576	42.0	9.80×10^5
X	577-670	31.2	3.61×10^5

of radioactivity was obtained having an R_f of 0.48 which corresponded to that of 16α-hydroxyandrostenedione. The eluate from the paper weighed 1.0 mg and contained 2.98×10^5 dpm. This was acetylated with ^{14}C-acetic anhydride solution No. 1, mixed with carrier 16α-acetoxyandrostenedione and percolated through a small alumina column. The residue obtained after elution with 60% benzene in Skellysolve B was crystallized to constant specific activity and the process was repeated after $NaBH_4$ reduction followed by DDQ oxidation as shown in Table 25. From the final $^3H/^{14}C$ ratio the specific activity of 16α-hydroxyandrostenedione was calculated as 1.84×10^6 dpm/mg.

Residue IV, Figure 12, Table 24, was chromatographed on a 100 g Celite column using system A and the distribution of radioactivity obtained

TABLE 25

Proof of Radiochemical Purity of 16α-Hydroxyandrostenedione isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Specific Activity (dpm ^3H/mg)

Crystallization	16α-Acetoxyandrostenedione				16α-Acetoxytestosterone			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	7300	24.8	5970	1.0	830	26.3	900	25.6
2	6910	22.6	6790	5.2	820	24.1	820	25.0
3	7020	25.3	6650	17.6				
4	7050	25.6	6900	26.0				
Calculated*	6540				880			

*The residue of pool III after purification was acetylated with acetic anhydride solution No. 1 and mixed with 21.0 mg of carrier 16α-acetoxyandrostenedione. The mixture was chromatographed on an alumina column to yield 13.5 mg of colorless oil containing 8.80×10^4 dpm ^3H. The fourth crystals and mother liquor were mixed with 23.0 mg carrier acetate reduced with $NaBH_4$ and the product oxidized with the DDQ reagent and then chromatographed on alumina to yield 23.4 mg of an oil which contained 2.10×10^4 dpm ^3H. The calculated specific activities were based on the residues obtained after column purification.

is shown in Figure 13. Pool IVA was chromatographed on four papers in system Q for 6 hours and one main radioactive peak was obtained at an average distance of 30.6 cm, the same as 16α-hydroxyandrosterone run alongside. Elution from the pepers gave 8.2 mg of crystals having 5.53×10^5 dpm. An infrared spectrum (KBr) of an aliquot of this material indicated that it contained a large amount of pregnanediol. Accordingly, it was rechromatographed on two papers in system W for 24 hours. The radioactive peak corresponding in mobility to 16α-hydroxyandrosterone was near the starting line. Elution yielded 2.3 mg of yellow oil containing 3.42×10^5 dpm. This oil was acetylated with ^{14}C-acetic anhydride solution No. 1 and the product chromatographed to a constant $^3H/^{14}C$ ratio using the Isotope Ratio Procedure as shown in Table 26. The specific activity of the metabolite in IVA, 16α-hydroxyandrosterone, was calculated to be 1.70×10^6 dpm/mg.

From IVB, Figure 13, a residue was obtained which was chromatographed on four papers in system Q for 7 hours. One radioactive peak was obtained at an average distance of 23.7 cm having the same mobility as 3α,16α-dihydroxyandrost-5-en-17-one. Elution from the papers gave 4.3 mg of crystalline material having 9.64×10^5 dpm and it was rechromatographed on paper in system W for 24 hours to remove any contaminating pregnanediol. The eluate was percolated through a 1 g silica gel column to yield 0.9 mg of colorless oil containing 7.64×10^5 dpm. This oil was acetylated with ^{14}C-acetic anhydride solution No. 1 and the product chromatographed to constant $^3H/^{14}C$ ratio as shown in Table 27. When constant $^3H/^{14}C$ ratio was attained after the first three systems employed, 3α,16α-diacetoxyandrost-5-en-17-one with both labels was added to allow for greater sensitivity in counting and the mixture was chromatographed to the predicted ratio as seen in the last two systems used. The specific activity of this metabolite, 3α,16α-dihydroxyandrost-5-en-17-one,

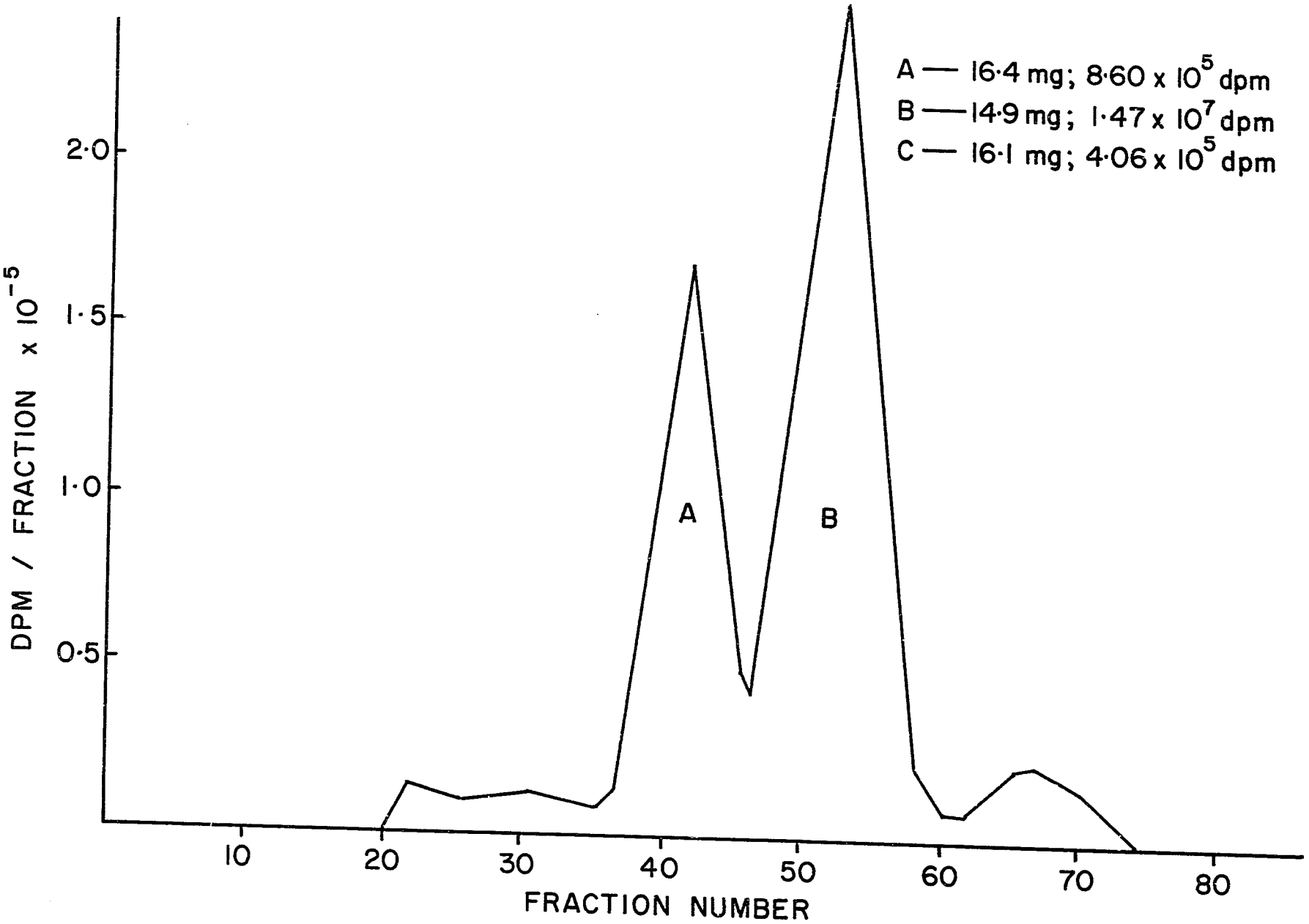

FIG. 13. Experiment 2. Celite column chromatography. Residue of Pool IV. Glucosiduronidate fraction.

TABLE 26

Proof of Radiochemical Purity of 16α-Hydroxyandrosterone Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

	Chromatographic System	Support	R_f or HBV	$^3H/^{14}C$ After Chromatography*			
				Proximal	Middle	Distal	Average
1	W	Paper	0.87	9.5	11.5	9.9	10.3
2	M	Celite	4	10.5	12.0	12.2	11.6
3	Benzene	Silica Gel	-	-	12.2	11.9	12.1
4	C	Celite	2,3	12.3	12.1	11.8	12.1
5	M	Celite	4,5	16.4	17.0	15.0	16.1
6	W	Paper	0.86	16.4	16.1	-	16.3

*The residue of IVA after purification contained 3.42×10^5 dpm. It was acetylated with acetic anhydride solution No. 1 and the product chromatographed in several systems. Aliquots from three sections of the radioactive peak were used to obtain the $^3H/^{14}C$ ratios. After the fourth chromatographic step the residue had 1.25×10^5 dpm ^3H and 1.03×10^4 dpm ^{14}C. This was mixed with 3α,16α-diacetoxy-5α-androstan-17-one containing 4.16×10^4 dpm ^3H, giving a calculated $^3H/^{14}C$ ratio of 16.2 and the mixture was rechromatographed in systems 5 and 6.

TABLE 27

Proof of Radiochemical Purity of 3α,16α-Dihydroxyandrost-5-en-17-one Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

	Chromatographic System	Support	R_f or HBV	$^3H/^{14}C$ After Chromatography*			
				Proximal	Middle	Distal	Average
1	C	Celite	3	-	22.5	20.6	21.6
2	W	Paper	0.72	21.9	21.8	20.7	21.5
3	60% Benzene in Skellysolve B	Alumina	-	20.2	22.2	21.5	21.3
4	C	Celite	2,3	12.8	12.8	12.4	12.7
5	M	Celite	4,5	12.7	12.9	12.7	12.8

*The residue of IVB after purification contained 5.14×10^5 dpm. It was acetylated with acetic anhydride solution No. 1 and the product chromatographed in several systems. Aliquots from three sections of the radioactive peak were used to obtain the $^3H/^{14}C$ ratios. After the third chromatographic step the residue had 1.14×10^5 dpm ^3H and 5.90×10^3 dpm ^{14}C. This was mixed with 3α,16α-diacetoxyandrost-5-en-17-one containing 4.62×10^4 dpm ^3H and 6.51×10^3 dpm ^{14}C to give a calculated $^3H/^{14}C$ ratio of 12.9 and the mixture was chromatographed in systems 4 and 5.

was calculated to be 3.02×10^6 dpm/mg.

The residue of IVC, Figure 13, was chromatographed on four papers in system T for 3 hours. One radioactive zone was observed with an R_f of 0.71 similar to 16α-hydroxydehydroisoandrosterone. On elution it gave 8.1 mg of crystals having 2.93×10^5 dpm. This was rechromatographed on paper in system W for 24 hours to remove any contaminating pregnanediol. Elution of the radioactive zone from the paper yielded 2.8 mg of yellow oil having 1.89×10^5 dpm. This was acetylated with ^{14}C-acetic anhydride solution No. 2 and the product mixed with 34.0 mg of carrier 3β,16α-diacetoxyandrost-5-en-17-one. The mixture was chromatographed on a small alumina column and then crystallized. Constant specific activity was achieved after the third crystallization and again after $NaBH_4$ reduction as shown in Table 28. The specific activity of the 16α-hydroxydehydroisoandrosterone was calculated to be 3.93×10^5 dpm/mg.

Pool VI, Figure 12, Table 24, was chromatographed on four papers in system Q for 7½ hours. One radioactive area was obtained at an average distance of 25.3 cm corresponding in mobility to 16α-hydroxyetiocholanolone. The eluate (19.6 mg and 8.00×10^5 dpm) was chromatographed on a small silica gel column to yield 2.1 mg of yellow oil which contained 7.02×10^5 dpm. This was acetylated with ^{14}C-acetic anhydride solution No. 1, and the product chromatographed to constant $^3H/^{14}C$ ratio as previously described. Constant $^3H/^{14}C$ ratio was attained after the third chromatographic procedure and again after the addition of ^3H-labelled carrier as shown in Table 29. The specific activity of this metabolite, 16α-hydroxyetiocholanolone, was calculated to be 1.19×10^6 dpm/mg.

The residue of pool IX, Figure 12, Table 24, was chromatographed on a 56 g Celite column in system K and the distribution of radioactivity obtained is shown in Figure 14. The contents of fractions 29-67 were combined to

TABLE 28

Proof of Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg)							
	3β,16α-Diacetoxyandrost-5-en-17-one				3β,16α-Diacetoxyandrost-5-en-17β-ol			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	2940	26.7	8520	59.0	2920	26.1	2940	26.8
2	2910	25.5	3130	26.5	2900	26.2	2940	25.8
3	2890	26.6	2900	25.9				
Calculated*	5550							

*The residue of IVC after purification was acetylated with acetic anhydride solution No. 2 and the product was mixed with 34.0 mg of carrier diacetate. The mixture was chromatographed on a small alumina column prior to crystallization. A total of 27.2 mg of crystalline material was eluted containing 1.52 x10^5 dpm ^3H and 3.70x10^3 dpm ^{14}C. These values were used to obtain the calculated specific activity. The third crystals and mother liquor were used in the formation of the 3β,16α-diacetoxyandrost-5-en-17β-ol.

TABLE 29

Proof of Radiochemical Purity of 16α-Hydroxyetiocholanolone Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

	Chromatographic System	Support	R_f or HBV	$^3H/^{14}C$ After Chromatography*			
				Proximal (-1)	Middle (0)	Distal (+1)	Average
1	W	Paper	0.82	4.6	5.6	3.6	4.6
2	M	Celite	3,4	7.9	8.3	-	8.1
3	Benzene	Alumina	-	-	8.2	8.7	8.5
4	C	Celite	2,3	12.9	13.2	12.9	13.0
5	W	Paper	0.83	12.7	12.5	12.3	12.5

*After purification the residue of pool VI was acetylated with acetic anhydride solution No. 1 and the product chromatographed several times. Aliquots from three sections of the radioactive peak were used to obtain the $^3H/^{14}C$ ratios. After the third chromatographic step 6.89×10^4 dpm ^3H and 7.90×10^3 dpm ^{14}C eluted from the column were mixed with 3.46×10^4 dpm ^3H 3α,16α-diacetoxy-5β-androstan-17-one to give a predicted ratio of 13.1, and the mixture was rechromatographed in systems 4 and 5.

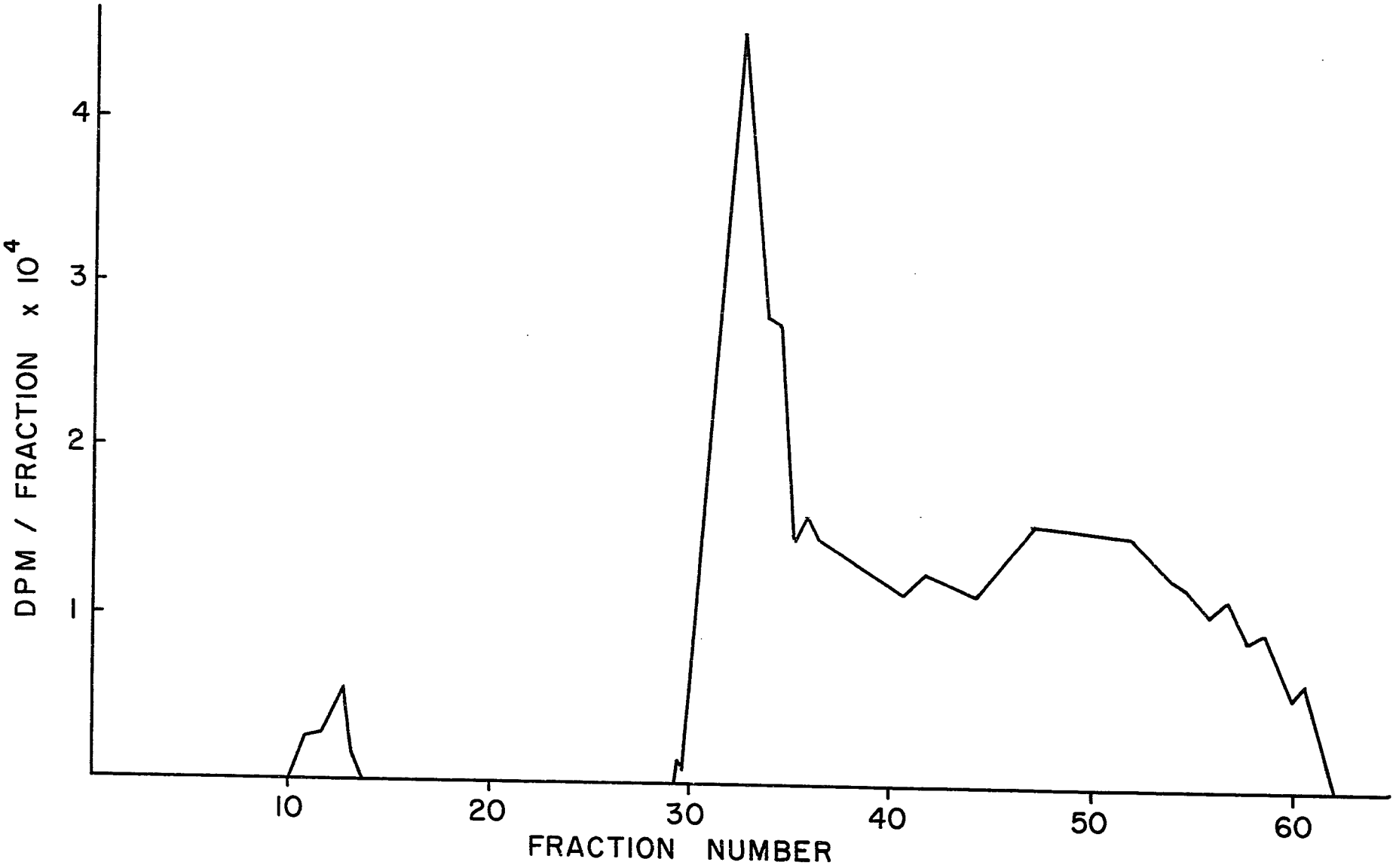

FIG. 14. Experiment 2. Celite column chromatography. Residue of Pool IX. Glucosiduronidate fraction. System K.

yield 27.7 mg of material containing 8.77×10^5 dpm. Crystallization from methanol afforded 14.0 mg of coarse needles: mp 274-276°C, mmp 276-277°C, specific activity 4.44×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of estriol. The remaining crystals were recrystallized as shown in Table 30. Constant specific activity was achieved after the second crystallization and again after the formation of the triacetate.

TABLE 30

Proof of Radiochemical Purity of Estriol Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

	Specific Activity (dpm/mg)			
	Estriol		Estriol triacetate	
Crystallization	Crystals	Mother Liquor	Crystals	Mother Liquor
1	44,430	18,340	31,220	29,860
2	43,180	43,990	30,410	31,030
Calculated*			30,870	

*The second crystals and mother liquor were used to prepare the triacetate. The calculated specific activity is based on the final specific activities of the estriol and molecular weight differences.

EXPERIMENT 3. Origin of 3α,16α-Dihydroxyandrost-5-en-17-one and the Metabolism of 16α-Hydroxyandrostenedione in Pregnancy

A normal 23-year old subject in the 34th week of pregnancy was injected intravenously with (7α-^{3}H)-16α-hydroxydehydroisoandrosterone (Lot # 2, 2.86×10^{7} dpm and 90 μg) and (4-^{14}C)-16α-hydroxyandrostenedione (2.51×10^{6} dpm and 60 μg), $^{3}H/^{14}C \simeq 11.5$. Urine was collected for five days and checked for completeness of collection by creatinine determinations (172) in the routine Endocrine laboratory. The steroid conjugates in each day's urine were simultaneously hydrolysed with Glusulase and a neutral extract containing the unconjugated steroids was prepared as previously described. Table 31 shows the ^{3}H and ^{14}C content of the extracts of each day's urine after correction for quenching.

TABLE 31

Radioactivity in the Extracts of Urine Following the Administration of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone and (4-^{14}C)-16α-Hydroxyandrostenedione

Day	^{3}H	% of Injected Dose	^{14}C	% of Injected Dose
1	1.99×10^{7}	69.6	1.64×10^{6}	65.6
2	1.28×10^{6}	4.3	1.05×10^{5}	4.2
3	6.72×10^{5}	2.3	6.68×10^{4}	2.6
4	1.62×10^{5}	0.5	1.82×10^{4}	0.7
5	6.40×10^{3}	-	5.60×10^{3}	-
Total	2.20×10^{7}	76.7	1.84×10^{6}	73.1

The total weight of the extracts from the five days was 2.1 g. It was chromatographed on a 280 g silica gel column using increasing concen-

trations of ethanol in methylene chloride as previously described. Figure 15 shows the radioactive material eluted from the column, and the mode of elution, weight and radioactivity of the residues from each pool are shown in Table 32.

TABLE 32

Elution of Radioactive Material from the Initial Silica Gel Column

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	^{14}C	$^{3}H/^{14}C$
I	239-280	246.1	4.31×10^{5}	3.76×10^{4}	11.4
II	281-340	58.8	9.13×10^{5}	2.56×10^{5}	3.6
III	341-380	27.6	9.45×10^{4}	9.10×10^{3}	10.4
IV	381-475	210.1	8.35×10^{6}	3.99×10^{5}	20.9
V	476-550	155.5	2.19×10^{6}	5.18×10^{5}	4.2
VI	551-715	291.1	5.91×10^{5}	6.91×10^{4}	9.6
VII	716-780	182.7	2.65×10^{6}	3.11×10^{5}	8.5
VIII	781-903	90.4	1.84×10^{6}	7.30×10^{4}	25.2

The labelled metabolites in Pools I, V and VIII have not as yet been identified.

Pool II was chromatographed on four papers in system P for six hours. One symmetrical zone of radioactivity was obtained with a mobility of 20.5 cm. The eluate was rechromatographed on two papers in system Q for 3 hours to yield one symmetrical zone of radioactivity having the same Rf (0.45) as that of 16α-hydroxyandrostenedione. The eluate from these papers contained 5.88×10^{5} dpm of ^{3}H and 1.61×10^{5} dpm of ^{14}C. Using Siiteri's method (165),

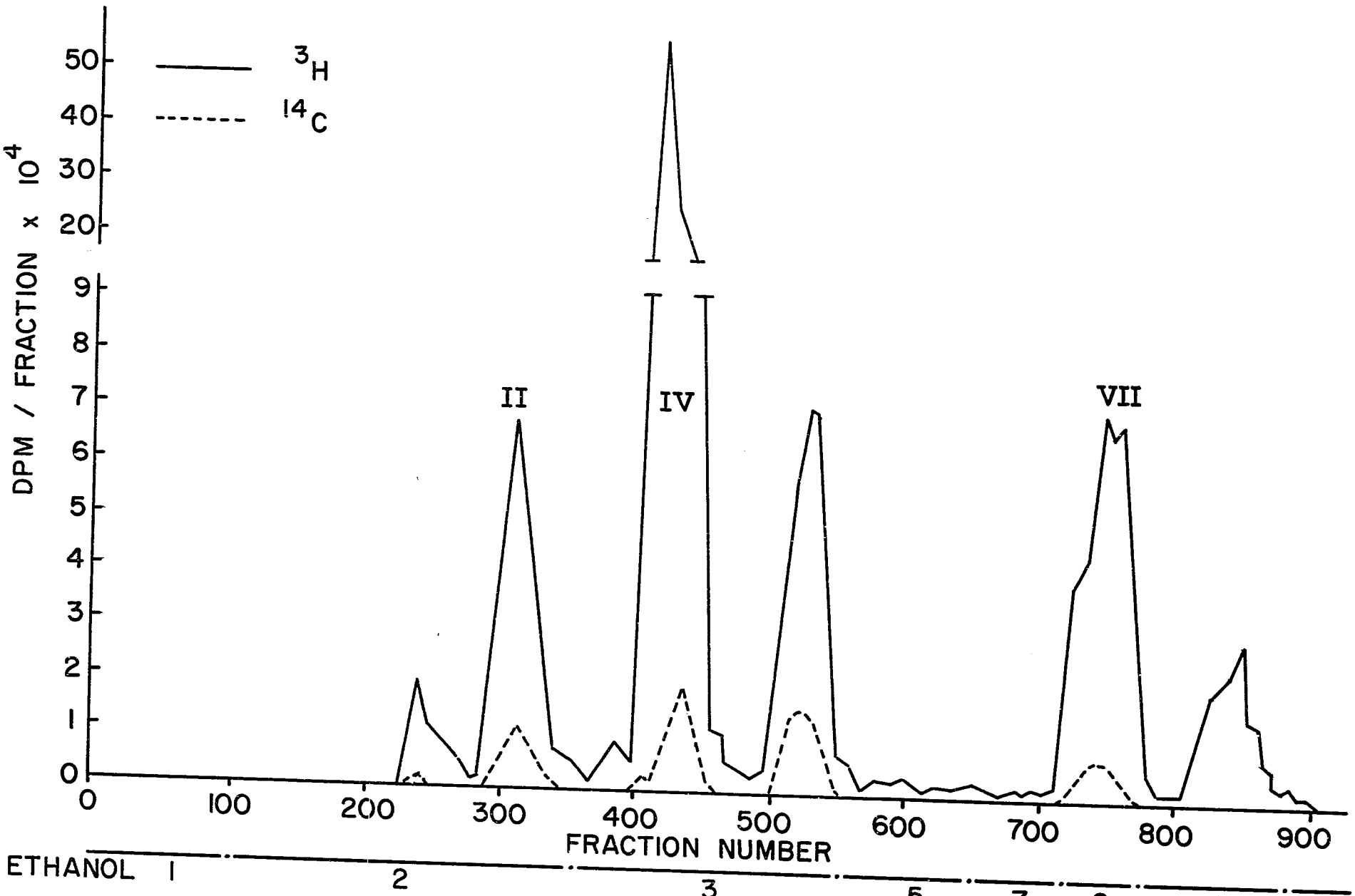

FIG. 15. Experiment 3. Silica gel chromatography.

equal aliquots were acetylated with non-labelled acetic anhydride and ^{3}H-acetic anhydride respectively, mixed with carrier 16α-acetoxyandrostenedione and percolated through small silica gel columns. Table 33 shows the specific activities and $^{3}H/^{14}C$ ratios obtained after crystallization of the acetates and the derivative, 16α-hydroxytestosterone. The calculated specific activity of the 16α-hydroxyandrostenedione was 5.18×10^{5} dpm ^{3}H/mg ($^{3}H/^{14}C$ = 3.2).

From pool IV, Figure 15, Table 32, a residue was obtained which was crystallized directly from methanol to give 60.2 mg of crystals which had the same infrared spectrum (KBr) as that of pregnanediol and virtually no radioactivity. The mother liquor (148.5 mg) was chromatographed on a 120 g Celite column using system A. Fractions of 10 ml each were collected at a rate of 20 ml per hour and a plot of radioactivity versus fraction number is shown in Figure 16.

The material within the radioactive peaks were pooled as follows: fractions 14 to 33, IVA; fractions 34-48, IVB; fractions 49-66, IVC; fractions 67-84, IVD. Pool IVC (30.9 mg, 5.43×10^{6} dpm ^{3}H and 3.85×10^{4} dpm ^{14}C) was chromatographed on a 30 g Celite column using system F and one main symmetrical peak of radioactivity was eluted in the 4th and 5th HBV. The material within this peak was combined to yield 6.5 mg of yellow oil having 5.25×10^{6} dpm ^{3}H and 2.0×10^{4} dpm ^{14}C. Chromatography on a 1 g alumina column afforded 3.7 mg of colorless oil which was eluted with 3% and 4% ethanol in benzene and could not be crystallized. One-tenth of this material was submitted to infrared analysis and the spectrum obtained (KBr) was identical to that of 3α,16α-dihydroxyandrost-5-en-17-one. The remaining material was acetylated with non-labelled acetic anhydride and chromatographed on several systems as shown in Table 34. After the third chromatographic step no ^{14}C could be detected in the metabolite. The infrared spectrum (KBr) of this material was identical

TABLE 33

Radiochemical Purity of 16α-Acetoxyandrostenedione after Acetylation of the Material in Pool II with Non-labelled and ^{3}H-labelled Acetic Anhydride

Crystallization	Specific Activity* (dpm ^{3}H/mg) Crystals	$^{3}H/^{14}C$	Mother Liquor	$^{3}H/^{14}C$	Specific Activity** (dpm ^{14}C/mg) Crystals	$^{3}H/^{14}C$	Mother Liquor	$^{3}H/^{14}C$	Specific Activity+ (dpm ^{3}H/mg) Crystals	$^{3}H/^{14}C$	Mother Liquor	$^{3}H/^{14}C$
1	5130	3.2	4830	3.2	1480	21.5	1650	85.4	2550	3.1	3180	3.4
2	4980	3.2	5050	3.2	1320	17.7	1460	32.5	2600	3.2	2490	3.1
3					1350	18.7	1480	21.2				
4					1320	18.8	1380	19.5				
Calculated	5130	3.2			1300				2680			

*After acetylation of ½ of the material in pool II with non-labelled acetic anhydride and chromatography of the product with carrier acetate on an alumina column, 34.0 mg of material was eluted containing 1.74×10^{5} dpm of ^{3}H and 5.41×10^{4} dpm of ^{14}C.

**The other ½ of the material in pool II was acetylated with ^{3}H-acetic anhydride (Batch #3), mixed with carrier acetate and chromatographed on an alumina column. A total of 32.0 mg of crystalline material was eluted containing 1.47×10^{7} dpm ^{3}H and 4.15×10^{4} dpm ^{14}C.

+ The crystals and mother liquor from the final crystallization of both types of 16α-acetoxyandrostenedione were combined and reduced with $NaBH_4$. The product was oxidized with DDQ and hydrolyzed. The hydrolysate was chromatographed on a small silica gel column and the material eluted at 1% and 2% ethanol in ether weighed 15.2 mg and contained 4.08×10^{4} dpm ^{3}H and 1.10×10^{4} dpm ^{14}C. These values were used to determine the calculated specific activity.

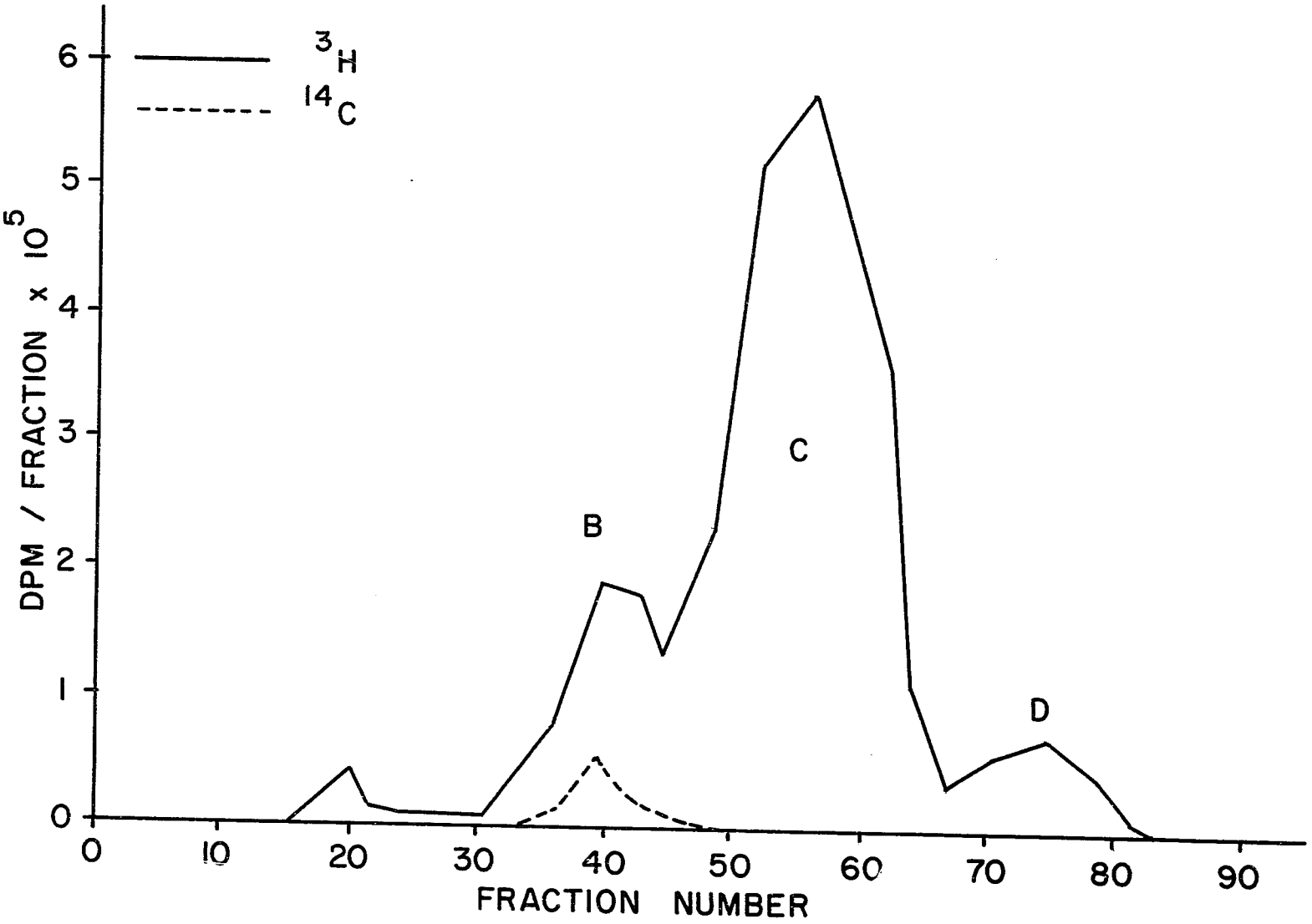

FIG. 16. Experiment 3. Celite column chromatography. Residue of pool IV. System A.

to that of 3α,16α-diacetoxyandrost-5-en-17-one. To obtain an accurate specific activity of this metabolite the aliquot taken for infrared analysis was recovered from the KBr disc, acetylated with ^{14}C-acetic anhydride solution No. 1 and the product was chromatographed in several systems as shown in Table 34. Constant $^{3}H/^{14}C$ ratio was achieved after the fifth chromatographic step and again after the addition of labelled carrier and rechromatography of the mixture (Steps 7 & 8, Table 34). On the basis of these data,

TABLE 34

Proof of Radiochemical Purity of 3α,16α-Dihydroxyandrost-5-en-17-one Isolated from Pool IVC of Pregnancy Urine

Chromatography*	Support	System	R_f or HBV	$^{3}H/^{14}C$ With ^{14}C-acetic anhydride	$^{3}H/^{14}C$ With non-labelled acetic anhydride
1	Alumina	Skellysolve B:Benzene (4:6)		-	255.0
2	Paper	W	0.72	8.9	-
3	Paper (reversed phase)	W	0.56	7.6	278,6
4	Celite	E	4	12.2	∞
5	Paper	M	0.50	14.2	∞
6	Celite	M	4-5	14.1	-
7	Celite	M	5	7.9	-
8	Paper	W	0.73	8.0	-
Calculated				7.8	

*Aliquots of the purified material IVC were acetylated with ^{14}C and non-labelled acetic anhydride and the products chromatographed. After the third chromatographic procedure the material acetylated with non-labelled acetic anhydride had no ^{14}C. After the fifth chromatographic procedure the material acetylated with ^{14}C-acetic anhydride contained 1.67×10^4 dpm ^{3}H and 1.10×10^3 dpm ^{14}C. This was mixed with 3α,16α-diacetoxyandrost-5-en-17-one containing 5.23×10^4 dpm ^{3}H and 7.82×10^3 dpm ^{14}C, to give a calculated $^{3}H/^{14}C$ of 7.8 and the mixture was chromatographed in systems 7 and 8.

-This procedure was not carried out.

the metabolite in pool IVC, 3α,16α-dihydroxyandrost-5-en-17-one, was shown to be radiochemically pure. Its specific activity was calculated to be 2.18×10^6 dpm ^3H/mg, and it did not contain any ^{14}C.

The residue of pool IVD, Figure 16 (13.0 mg, 7.21×10^5 dpm ^3H and 1.96×10^4 dpm ^{14}C) was chromatographed on one paper in system P for 28 hours. One radioactive peak was obtained with a mobility of 16.7 cm which corresponded to that of 16α-hydroxydehydroisoandrosterone run alongside. The eluate weighed 5.6 mg and contained 3.69×10^5 dpm ^3H and 1.04×10^4 dpm ^{14}C. An aliquot containing 7.38×10^4 dpm ^3H and 2.08×10^3 dpm ^{14}C was mixed with 41.3 mg carrier 16α-hydroxydehydroisoandrosterone and crystallized to constant specific activity and again after the formation of a derivative as shown in Table 35. The rest of the purified material was percolated through a 1 g silica gel column and crystallized from acetone:Skellysolve B to yield 3.0 mg of fine needles. Its infrared spectrum (KBr) was identical to that of 16α-hydroxydehydroisoandrosterone: mp 178-180°C, mmp 179-181°C. An aliquot containing 7.89×10^5 dpm ^3H was acetylated with ^{14}C-acetic anhydride solution No.2 and mixed with carrier acetate. The mixture was percolated through a small alumina column and crystallized to constant specific activity and again after $NaBH_4$ reduction as shown in Table 36. The calculated specific activity of 16α-hydroxydehydroisoandrosterone was 9.13×10^4 dpm ^3H/mg.

The residue of pool VII, Figure 15, Table 32 was chromatographed on a 140 g Celite column using system B (HBV = 200 ml) and one main radioactive peak was obtained in the 3rd HBV. The material within this peak was combined to yield 88.9 mg of brown oily material containing 2.10×10^6 dpm ^3H and 1.99×10^5 dpm ^{14}C. This material was crystallized directly from methanol and methanol-ether as shown in Table 37. The infrared spectrum (KBr) of the third crystals was identical to that of estriol: mp 280-282°C, mmp 279-280°C. The third crystals and mother liquor were combined and acetylated with

TABLE 35

Proof of Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated After the Administration of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone and 4-^{14}C-16α-Hydroxyandrostenedione to a Pregnant Female

	Specific Activity (dpm ^{3}H/mg)							
	16α-Hydroxydehydroisoandrosterone				3β,16α-Diacetoxyandrost-5-en-17-one			
Crystallization	Crystals	^{3}H/^{14}C	Mother Liquor	^{3}H/^{14}C	Crystals	^{3}H/^{14}C	Mother Liquor	^{3}H/^{14}C
1	1730	71.9	1990	25.9	1260	∞	1160	131.2
2	1670	79.7	1740	38.4	1220	∞	1220	∞
3	1660	106.1	1610	50.6	1200	∞	1190	∞
4	1640	90.1	1580	52.6				
5	1440	203.0	1570	84.3				
6	1450	312.8	1440	100.8				
Calculated*	1790				1130			

*A total of 7.38×10^{4} dpm ^{3}H and 2.08×10^{3} dpm ^{14}C of purified material from Pool IVD was mixed with 41.3 mg of carrier 16α-hydroxydehydroisoandrosterone and crystallized. The sixth crystals and mother liquor were used to prepare the diacetate which was chromatographed on a small alumina column prior to crystallization.

TABLE 36

Proof of Radiochemical Purity of 3β,16α-Diacetoxyandrost-5-en-17-one Prepared from the Residue of IVD After the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone and (4-^{14}C)-16α-Hydroxyandrostenedione to a Pregnant Female

	Specific Activity (dpm ^{3}H/mg)							
	3β,16α-Diacetoxyandrost-5-en-17-one				3β,16α-Diacetoxyandrost-5-en-17β-ol			
Crystallization	Crystals	^{3}H/^{14}C	Mother Liquor	^{3}H/^{14}C	Crystals	^{3}H/^{14}C	Mother Liquor	^{3}H/^{14}C
1	2290	6.3	2590	3.8	2320	6.7	2240	7.1
2	2310	6.5	2450	5.2	2410	6.9	2290	6.9
3	2290	6.3	2270	6.4				
Calculated*	2290							

*An aliquot of purified IVD containing 7.89x10^{5} dpm ^{3}H was acetylated with ^{14}C-acetic anhydride solution No. 2 and then mixed with 34.0 mg carrier diacetate. The mixture was chromatographed on a small alumina column and a total of 29.0 mg of crystalline material was eluted containing 6.65x10^{4} dpm ^{3}H and 1.27x10^{4} dpm ^{14}C. These values were used to determine the calculated specific activity. The third crystals and mother liquor were reduced with NaBH4 and the product was chromatographed on a small alumina column prior to crystallization.

with non-labelled acetic anhydride. After chromatography of the product on a small alumina column and crystallization of the material eluted, the $^{3}H/^{14}C$ ratio and specific activities were constant and corresponded to the expected values as is evident from the data in Table 37.

TABLE 37

Proof of Radiochemical Purity of Estriol Isolated After the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone and (4-^{14}C)-16α-Hydroxyandrostenedione to a Pregnant Female

Crystallization	Specific Activity (dpm ^{3}H/mg) Estriol				Estriol triacetate			
	Crystals	$^{3}H/^{14}C$	Mother Liquor	$^{3}H/^{14}C$	Crystals	$^{3}H/^{14}C$	Mother Liquor	$^{3}H/^{14}C$
1	36,700	10.2	9,400	9.7	24,700	9.8	21,500	10.0
2	38,100	10.1	34,900	10.4	24,800	10.0	25,100	10.0
3	36,500	10.0	36,500	10.1				
Calculated*	23,700				25,400			

*After purification of the residue from pool VII, Figure 15, 88.9 mg of material (2.10×10^{6} dpm ^{3}H and 1.99×10^{5} dpm ^{14}C) was obtained. The calculated specific activity of the estriol was based on these values while that of the estriol triacetate was computed from the final crystallization of estriol and the altered molecular weight.

EXPERIMENT 4. Formation of 16α-Hydroxysteroids in Late Pregnancy.

A mixture of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone (Lot # 2, 2.24x 10^{7} dpm in 71 μg) and (4-^{14}C)-dehydroisoandrosterone sulfate (4.37x10^{6} dpm in 28 μg), $^{3}H/^{14}C$ = 5.1, was injected intravenously into a normal 23-year old subject in the 36th week of pregnancy. Urine was collected for five days and a creatinine determination carried out on each day's urine. The steroid conjugates in each 24-hour urine were first solvolysed and then hydrolysed with β-glucuronidase as previously described. The radioactivity in the individual neutral extracts obtained after hydrolysis is shown in Table 38.

The combined extracts from the sulfate fraction weighed 850 mg and were chromatographed on a 200 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described, and the elution of radioactive material is shown in Figure 17. The mode of elution, the weight and radioactivity of each pool are shown in Table 39.

TABLE 39

Radioactive Material Eluted after Silica Gel Chromatography of the Sulfate Fraction of Pregnancy Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	^{14}C	$^{3}H/^{14}C$
I	179-196	166.8	3.92x10^{5}	2.71x10^{5}	1.34
II	260-309	41.4	1.01x10^{5}	4.86x10^{4}	2.07
III	310-358	70.3	7.52x10^{5}	2.41x10^{5}	3.12
IV	359-362	61.1	1.52x10^{5}	7.03x10^{4}	2.16
V	442-525	103.1	2.32x10^{5}	9.79x10^{4}	2.37
VI	526-610	33.7	4.82x10^{5}	1.07x10^{5}	4.51

The residue of pool III was chromatographed on a 70 g Celite column

TABLE 38

Radioactivity in the Sulfate and Glucosiduronidate Fractions of Pregnancy Urine

Day	Sulfates				Glucosiduronidates			
	^{3}H	% of injected dose	^{14}C	% of injected dose	^{3}H	% of injected dose	^{14}C	% of injected dose
1	2.13×10^{6}	9.51	7.38×10^{5}	16.88	1.40×10^{7}	62.47	8.43×10^{5}	19.29
2	1.95×10^{5}	0.81	1.45×10^{5}	3.32	8.95×10^{5}	3.99	3.20×10^{5}	7.33
3	4.64×10^{4}	0.02	5.36×10^{4}	1.23	2.46×10^{5}	1.11	1.77×10^{5}	4.05
4	3.49×10^{4}	0.01	2.31×10^{4}	0.53	1.36×10^{5}	0.61	1.72×10^{5}	3.93
5	2.97×10^{4}	0.01	1.24×10^{4}	0.28	1.06×10^{5}	0.48	4.36×10^{4}	1.00
Total	2.44×10^{6}	10.41	8.41×10^{5}	22.28	1.54×10^{7}	68.46	1.56×10^{6}	35.60

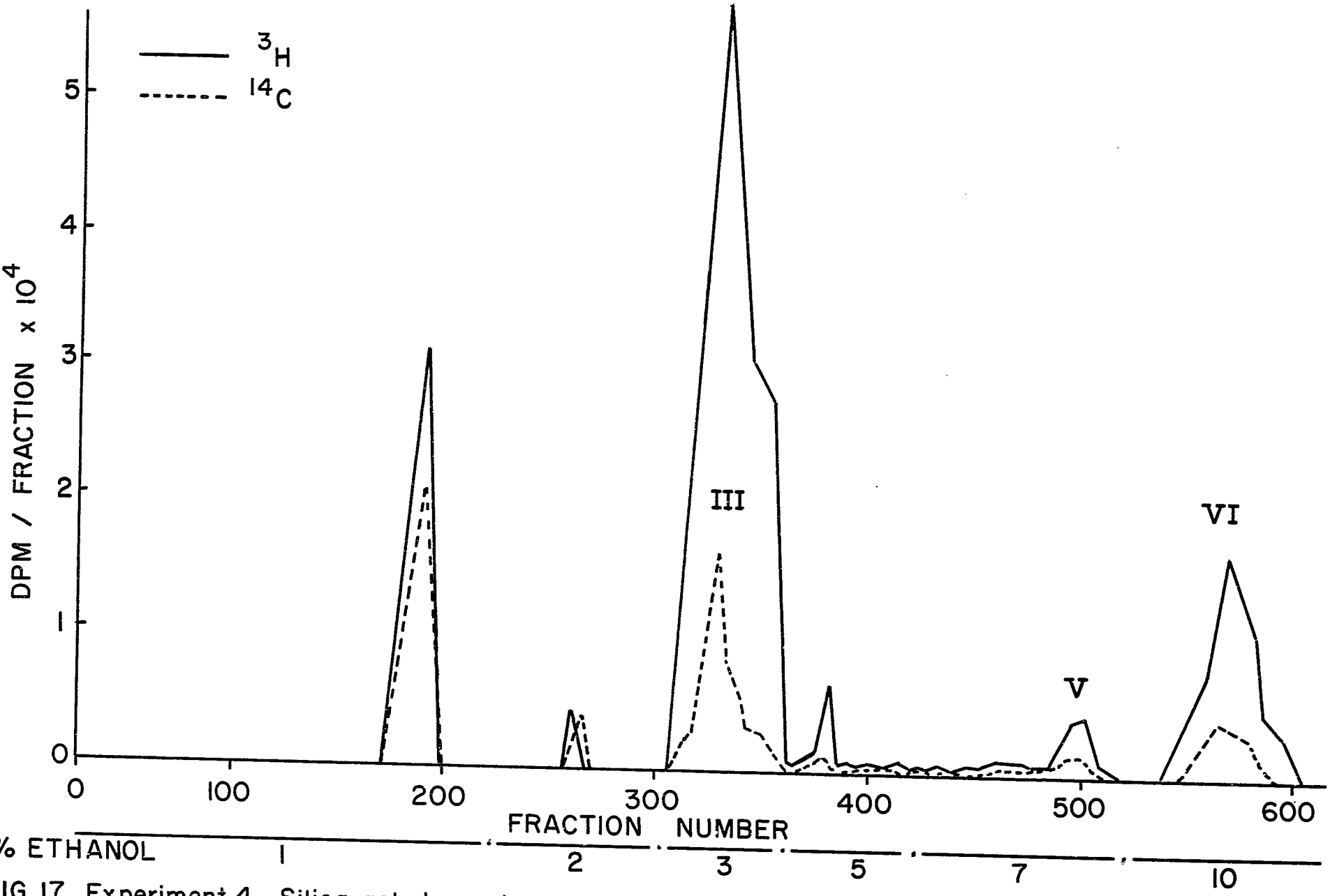

FIG. 17. Experiment 4. Silica gel chromatography of sulfate fraction.

using system A (HBV = 90 ml). The material in the 2nd HBV (IIIA), 3rd and 4th HBV (IIIB), and 5th and 6th HBV (IIIC) were combined separately. Pool IIIB (6.5 mg, 2.39×10^5 dpm ^3H and 2.71×10^4 dpm ^{14}C) was chromatographed on 2 papers in system Q for 7 hours and the radioactive zone at 28.7 cm corresponding in mobility to 3α,16α-dihydroxyandrost-5-en-17-one was eluted. The eluate (2.9 mg, 6.12×10^4 dpm ^3H and 4.10×10^3 dpm ^{14}C) was successively chromatographed on paper in system P and system Q for 5 hours, but the radioactivity remained at the starting line on both occasions.

The crystalline residue of pool IIIC weighed 17.7 mg and contained 5.36×10^5 dpm ^3H and 1.81×10^5 dpm ^{14}C. It was chromatographed on a small silica gel column and elution with 2% ethanol in methylene chloride yielded 12.0 mg of material having 4.88×10^5 dpm ^3H and 1.63×10^5 dpm ^{14}C. This material was crystallized to constant specific activity and again after the formation of the diacetate as shown in Table 40. The infrared spectrum (KBr) of the third crystals of the diol was identical to that of 16α-hydroxydehydroisoandrosterone: mp 179-180°C, mmp 178-180°C.

From pool V, Figure 17, Table 39, a residue was obtained which was chromatographed on a 50 g Celite column in system K (HBV = 70 ml) and the material eluted in the 4th and 5th HBV was combined to yield 7.2 mg of residue having 1.32×10^5 dpm ^3H and 5.94×10^4 dpm ^{14}C. Crystallization from methanol afforded 2.5 mg of fine needles, mp 277-279°C, mmp 278-280°C, specific activity 2.37×10^4 dpm ^3H/mg, $^3H/^{14}C$ = 3.1. Its infrared spectrum (KBr) was identical to that of estriol. An aliquot of the crystals was mixed with carrier estriol and crystallized. Constant specific activity was achieved after the second crystallization and again after the formation of a derivative as shown in Table 41.

The residue of pool VI, Figure 17, Table 39, was crystallized twice from methanol and ethanol-methanol. The specific activity of the first

TABLE 40

Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated from the Sulfate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg) 16α-Hydroxydehydroisoandrosterone Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	3β,16α-Diacetoxyandrost-5-en-17-one Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	41,200	3.0	50,500	4.0	3,230	2.8	3,250	2.5
2	39,500	2.7	41,300	3.2	3,190	2.6	3,220	2.7
3	41,400	2.7	39,500	2.6				
Calculated*					3,160			

*An aliquot from the third crystals was acetylated with non-labelled acetic anhydride and the product was mixed with carrier diacetate. The mixture was chromatographed on a small silica gel column to yield 13.8 mg of crystalline material having 4.35×10^4 dpm ^3H and 1.69×10^4 dpm ^{14}C. These values were used to determine the calculated specific activity of the diacetate.

TABLE 41

Proof of Radiochemical Purity of Estriol Isolated from the Sulfate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
Estriol				
1	1570	3.1	1520	2.8
2	1520	2.7	1530	2.7
Calculated*	1650			
Estriol triacetate				
1	1180	2.9	1030	2.8
2	1200	2.6	1120	2.8
Calculated*	1010			

*A total of 2.08×10^4 dpm ^3H of crystalline material from pool V was mixed with 12.6 mg of estriol carrier.

crystals was 4.85×10^4 dpm ^3H/mg, $^3H/^{14}C$ = 3.3, while those of the second crystals and mother liquor were 4.71×10^4 dpm ^3H/mg, $^3H/^{14}C$ = 3.2 and 4.66×10^4 dpm ^3H/mg, $^3H/^{14}C$ = 3.2 respectively. The mp of the second crystals was 267-269°C and its infrared spectrum (KBr) was identical to that of Hirschmann's triol.

The combined glucosiduronidate fraction from the five days (2.6 g) was chromatographed on a 325 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. A plot of radioactivity versus fraction number is shown in Figure 18. The mode of elution, the weight and radioactivity of each pool are shown in Table 42. The residues in pools I, II, V, VI and VIII are currently under investigation.

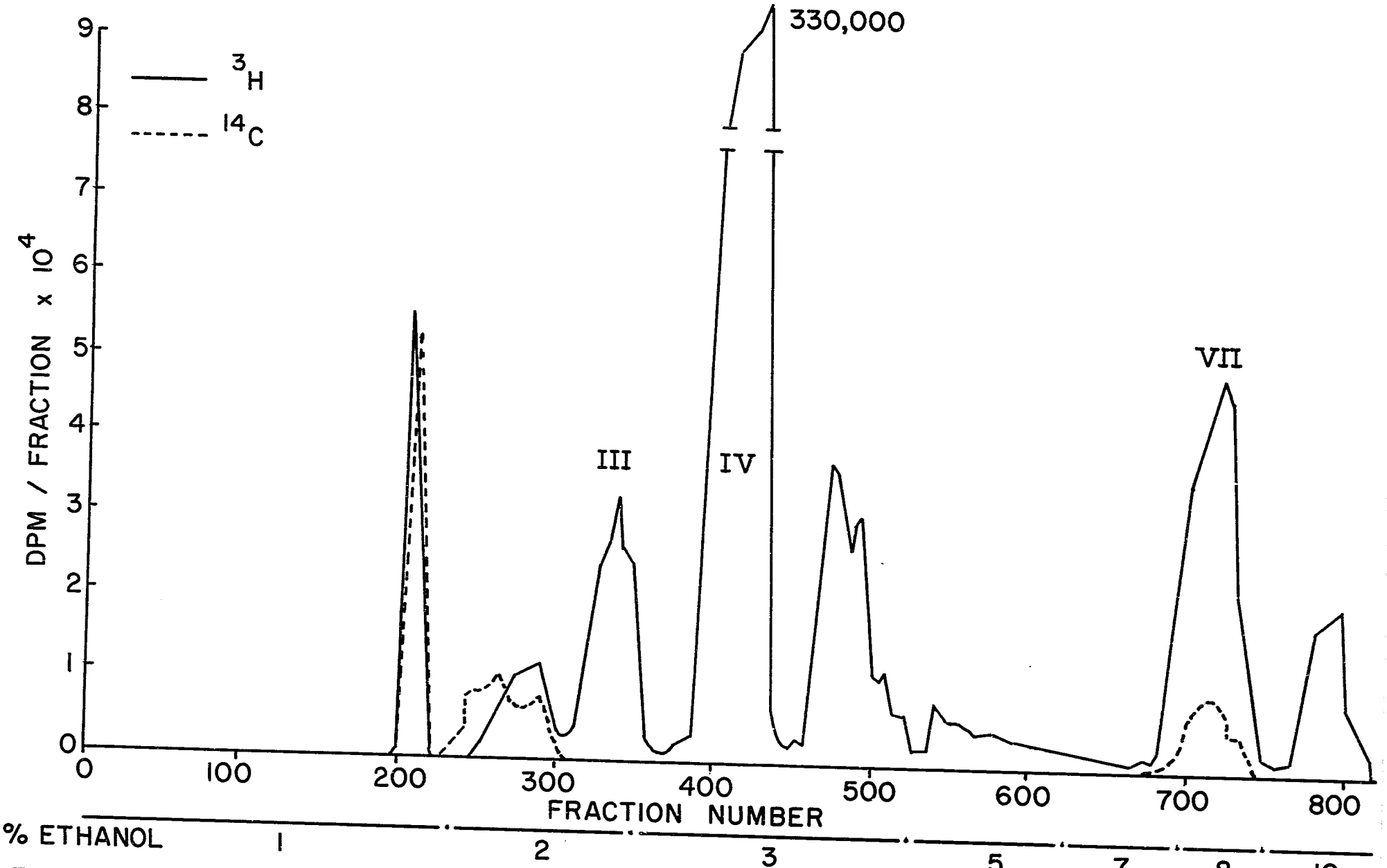

FIG. 18. Experiment 4. Silica gel chromatography of glucosiduronidate fraction.

TABLE 42

Radioactive Material Eluted after Silica Gel Column Chromatography of the Glucosiduronidate Fraction of Pregnancy Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	^{14}C	$^{3}H/^{14}C$
I	197-240	112.8	$3.61x10^{5}$	$3.65x10^{5}$	0.99
II	241-300	48.6	$2.58x10^{5}$	2.41x105	1.07
III	301-355	70.9	$7.18x10^{5}$	$1.82x10^{4}$	39.46
IV	356-440	1039.2	$5.78x10^{6}$	$1.06x10^{5}$	54.42
V	441-525	264.3	$1.76x10^{6}$	$7.70x10^{4}$	22.79
VI	526-670	186.6	$8.15x10^{5}$	$7.92x10^{4}$	10.28
VII	671-765	172.8	$1.89x10^{6}$	$2.68x10^{5}$	7.04
VIII	766-830	66.1	$6.94x10^{5}$	$6.30x10^{4}$	11.02

From pool III, Figure 18, Table 42, a residue was obtained which was chromatographed on 4 papers in system N for 29 hours. One radioactive area was observed at an average distance of 18.5 cm corresponding in mobility to 16α-hydroxyandrostenedione. The eluate was chromatographed on one paper in system Q and one radioactive area was obtained with an R_f of 0.50 which corresponded to that of 16α-hydroxyandrostenedione. One half of the eluted material which contained $5.13x10^{5}$ dpm ^{3}H and $3.70x10^{3}$ dpm ^{14}C was acetylated with non-labelled acetic anhydride and the other half was acetylated with ^{14}C-acetic anhydride solution "A". The individual products were mixed with carrier 16α-acetoxyandrostenedione and purified by chromatography on small alumina columns prior to crystallization to constant specific activity as shown in Table 43. From the corrected $^{3}H/^{14}C$ ratio the specific activity of

TABLE 43

Proof of Radiochemical Purity of 16α-Acetoxyandrostenedione Prepared from Pool III of the Glucosiduronidate Fraction of Pregnancy Urine

	Specific Activity (dpm ^3H/mg)							
	½ of 16α-Hydroxyandrostenedione Acetylated with non-labelled acetic anhydride				½ of 16α-Hydroxyandrostenedione acetylated with ^{14}C-acetic anhydride			
Crystallization	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	10,420	172	14,070	135	7,690	3.8	11,130	0.1
2	9,310	183	12,160	155	7,890	3.6	7,560	1.3
3	8,970	192	9,170	189	8,070	3.5	7,960	2.9
4					7,950	3.7	7,740	3.7
Calculated*	9,880				8,120			

*Identical aliquots of purified material from Pool III were acetylated with non-labelled acetic anhydride and ^{14}C-acetic anhydride solution "A". The individual products were mixed with carrier 16α-acetoxyandrostenedione and chromatographed on small alumina columns prior to crystallization.

Corrected $^3H/^{14}C$ = 3.6

Calculated specific activity of 16α-hydroxyandrostenedione = 5.14×10^5 dpm ^3H/mg.

the 16α-hydroxyandrostenedione was calculated to be 5.14×10^5 dpm ^3H/mg. In this regard the specific activity of this metabolite with respect to ^{14}C may not be meaningful because of the high $^3H/^{14}C$ ratios. A derivative was not prepared because it had been shown previously (Tables 25 and 33) that this procedure provides radiochemically pure 16α-hydroxyandrostenedione after the first set of crystallizations.

Direct crystallization of the material in Pool IV, Figure 18 was necessa to remove the pregnanediol present. The mother liquor (890 mg) was chromatographed on a 200 g Celite column using system A (HBV = 270 ml). The material contained in fractions 20-56 (IVA), 57-95 (IVB) and 96-118 (IVC) were combined. The residue of pool IVB which weighed 49.8 mg and contained 1.71×10^6 dpm ^3H and 1.00×10^4 dpm ^{14}C was chromatographed on a 50 g Celite column using system F (HBV = 80 ml). One main radioactive peak was obtained in the 5th and 6th HBV and the material within this peak was combined to yield 18.8 mg of yellow oil containing 1.33×10^6 dpm ^3H and 5.45×10^3 dpm ^{14}C. It was chromatographed on paper in system P for 18 hours and one radioactive pea was obtained at a distance of 24.2 cm from the starting line, corresponding in mobility to 3α,16α-dihydroxyandrost-5-en-17-one. Accordingly, identical aliquots were acetylated with non-labelled acetic anhydride and ^{14}C-acetic anhydride solution No. 1 and the individual products were chromatographed several times as previously described. In Table 44 are shown the various systems employed and the $^3H/^{14}C$ ratio obtained. After the first chromatographic step the acetate formed from non-labelled acetic anhydride contained no ^{14}C. From the data shown in Table 44 it was possible to assign the structure of 3α,16α-dihydroxyandrost-5-en-17-one to the metabolite in pool IVB. The specific activity of this metabolite was calculated to be 1.72×10^6 dpm/mg.

TABLE 44

Proof of Radiochemical Purity of 3α,16α-Dihydroxyandrost-5-en-17-one Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Chromatography*	Support	System	Rf or HBV	$^3H/^{14}C$ After ^{14}C-Acetic anhydride	$^3H/^{14}C$ After Acetylation with Non-labelled acetic anhydride
1	Paper	W	0.73	4.1	327.0
2	Paper	M	0.52	7.1	∞
3	Celite	E	4	9.2	∞
4	Celite	M	4,5	12.2	-
5	Celite	C	3	12.2	-
6	Celite	M	5	8.3	
7	Paper	W	0.72	8.4	

*After the fifth chromatographic procedure the residue contained 2.21×10^4 dpm ^3H and 1.75×10^3 dpm ^{14}C. To this was added 3α,16α-diacetoxyandrost-5-en-17-one containing 5.87×10^4 dpm ^3H and 8.36×10^3 dpm ^{14}C to give an $^3H/^{14}C$ ratio of 8.0

-Not carried out.

The residue of pool IVC (5.6 mg, containing 1.89×10^5 dpm ^3H and 1.40×10^3 dpm ^{14}C) was chromatographed on paper in system P for 26 hours. One radioactive peak was obtained with a mobility of 19.5 cm corresponding to that of 16α-hydroxydehydroisoandrosterone and the eluate weighed 1.9 mg, and contained 3.22×10^4 dpm ^3H and 3.00×10^2 dpm ^{14}C. One half was mixed with carrier 16α-hydroxydehydroisoandrosterone and crystallized as shown in Table 45. The other half was acetylated with ^{14}C-acetic anhydride solution No. 1 and mixed with carrier acetate. The mixture was purified by chromatography on a small silica gel column and crystallized as shown in Table 46. From the final $^3H/^{14}C$ ratios, the specific activity of 16α-hydroxydehydroisoandrosterone was calculated to be 1.62×10^6 dpm/mg.

TABLE 45

Proof of Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg)							
	16α-Hydroxydehydroisoandrosterone				3β,16α-Diacetoxyandrost-5-en-17-one			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	530	105	810	70	400	135	420	260
2	500	137	540	86	390	∞	400	∞
3	500	130	490	132				
Calculated*	610				400			

*A total of 3.20×10^4 dpm ^3H and 150 dpm ^{14}C was mixed with 15.0 mg of carrier 16α-hydroxydehydroisoandrosterone and the mixture was chromatographed on a small silica gel column. A total of 13.7 mg of material was eluted having 8.35×10^3 dpm ^3H. The third crystals and mother liquor were used to prepare the diacetate. The product, after acetylation, was mixed with 5.1 mg carrier diacetate and the mixture was chromatographed on a small alumina column prior to crystallization.

TABLE 46

Proof of Radiochemical Purity of 3β,16α-Diacetoxyandrost-5-en-17-one Prepared from the Material in Pool IVC of the Glucosiduronidate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg) 3β,16α-Diacetoxyandrost-5-en-17-one				3β,16α-Diacetoxyandrost-5-en-17β-ol			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	590	3.3	1340	0.3	600	11.8	580	12.0
2	640	8.1	1110	0.5	610	12.0	610	11.6
3	640	11.0	930	6.7				
4	630	12.0	640	11.2				
Calculated*	970							

*After acetylation of an aliquot of purified IVC with acetic anhydride solution No. 1 the product was mixed with 10.3 mg carrier acetate and chromatographed on a small silica gel column. A total of 9.7 mg of crystalline material containing 9.40×10^4 dpm ^3H and 2.29×10^4 dpm ^{14}C was eluted. The fourth crystals and mother liquor were reduced with $NaBH_4$ and the product chromatographed on a small alumina column prior to crystallization.

From pool VII, Figure 18, Table 42, a residue was obtained which was chromatographed on a 140 g Celite column using system K and the radioactivity eluted versus fraction number is shown in Figure 19. Fractions 117-180 were combined to yield 55.8 mg of material containing 1.40×10^6 dpm ^3H and 2.00×10^5 dpm ^{14}C. This material was crystallized twice from methanol and methanol-ether as shown in Table 47. The mp of the second crystals was 280-281°C. Its infrared spectrum (KBr) was identical to that of estriol.

TABLE 47

Radiochemical Purity of Estriol Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg) Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
Estriol				
1	33,350	7.1	8,890	7.7
2	32,180	7.3	31,610	7.3
Estriol triacetate				
1	22,340	7.4	17,410	7.0
2	21,860	7.4	21,790	7.5
Calculated*	22,180			

*The second crystals and mother liquor of the estriol were used to prepare the triacetate. The calculated specific activity was based on the final specific activities of the estriol and altered molecular weight.

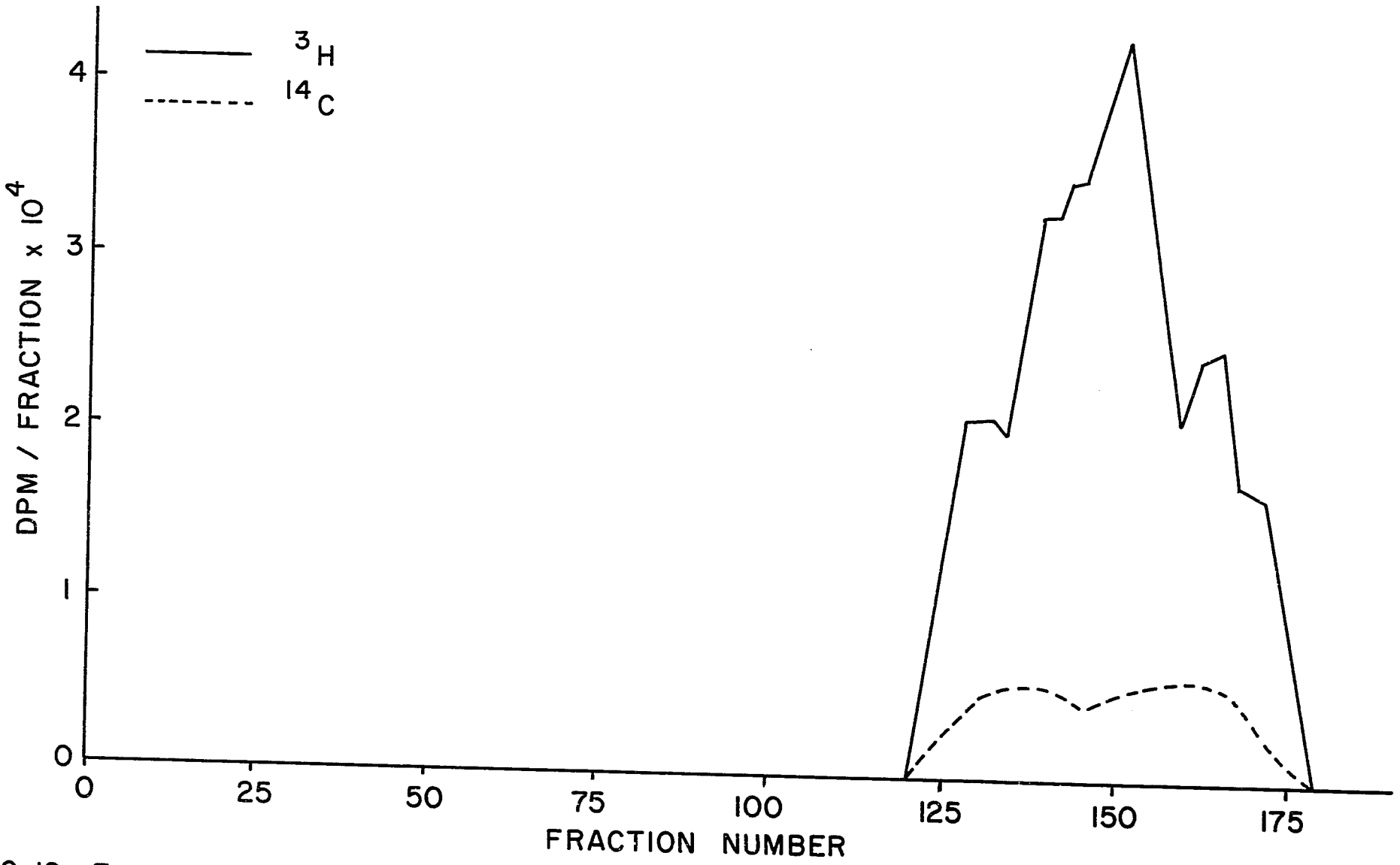

FIG. 19. Experiment 4. Celite column chromatography. Residue of pool VII. Glucosiduronidate fraction. System K.

EXPERIMENT 5. Metabolism of 16α-Hydroxydehydroisoandrosterone-3-sulfate and Dehydroisoandrosterone sulfate in Late Pregnancy

A normal 25-year old subject near term was injected intravenously with a mixture of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone-3-sulfate (Lot #2, 2.20×10^{7} dpm in 3.5 μg) and (4-^{14}C)-dehydroisoandrosterone sulfate (2.12×10^{6} dpm in 8.9 μg), $^{3}H/^{14}C$ = 10.4. Two 24-hour urine samples were collected before labour commenced. The conjugates in the urines were hydrolysed to give a sulfate fraction and a glucosiduronidate fraction as previously described. Table 48 shows the radioactivity in each day's hydrolysate. The processing of these extracts is described below.

TABLE 48

Radioactivity in the Sulfate and Glucosiduronidate Extracts of Pregnancy Urine

Day	^{3}H	% of injected dose	^{14}C	% of injected dose
Sulfates				
1	5.46×10^{6}	24.8	2.00×10^{5}	9.5
2	1.32×10^{6}	6.0	9.13×10^{4}	4.3
Glucosiduronidates				
1	5.27×10^{6}	23.9	4.34×10^{5}	20.1
2	1.14×10^{6}	5.2	1.78×10^{5}	8.4
Total	13.19×10^{6}	59.9	9.03×10^{5}	42.3

Some time later, a mixture of the same substrates ($^{3}H/14C$ = 7.1), was injected intravenously to a subject in the 40th week of pregnancy and the radioactivity obtained in the individual sulfate and glucosiduronidate fractions of the urine is shown in Table 49. These extracts are currently under investigation and are not described in this thesis.

TABLE 49

Radioactivity in the Sulfate and Glucosiduronidate Fractions of Pregnancy Urine after the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone-3-sulfate and (4-^{14}C)-Dehydroisoandrosterone-3-sulfate ($^{3}H/^{14}C$ = 7.1)

	Sulfates				Glucosiduronidates			
Day	^{3}H	% of Injected Dose	^{14}C	% of Injected Dose	^{3}H	% of Injected Dose	^{14}C	% of Injected Dose
1	2.96×10^{6}	10.89	3.00×10^{5}	7.80	5.88×10^{6}	21.63	7.49×10^{5}	19.45
2	6.89×10^{5}	2.53	8.98×10^{4}	2.33	2.40×10^{6}	8.85	4.31×10^{5}	11.20
3	6.68×10^{4}	0.24	9.20×10^{3}	0.23	3.98×10^{5}	1.46	7.78×10^{4}	2.02
4	3.44×10^{4}	0.12	1.09×10^{4}	0.28	8.85×10^{4}	0.40	1.44×10^{4}	0.37
5	7.00×10^{3}	0.03	1.20×10^{3}	0.03	3.12×10^{4}	0.11	5.70×10^{3}	0.14
6	9.55×10^{3}	0.04	1.90×10^{3}	0.05	2.45×10^{4}	0.09	3.80×10^{3}	0.09
Total	3.77×10^{6}	13.85	4.13×10^{5}	10.72	8.83×10^{6}	32.54	1.28×10^{6}	33.27

The combined sulfate fraction, 421 mg, was chromatographed on a 120 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described, and the elution of radioactivity is shown in Figure 20. The mode of elution, the weight and radioactivity of each pool are shown in Table 50.

TABLE 50

Elution of Radioactive Material after Silica Gel Column Chromatography of the Sulfate Fraction of Pregnancy Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	Radioactivity (dpm) ^{14}C
I	115-130	37.0	5.86×10^{4}	3.01×10^{4}
II	131-159	6.5	7.47×10^{4}	1.22×10^{4}
III	160-212	17.1	2.49×10^{5}	3.75×10^{4}
IV	213-291	36.0	3.32×10^{6}	9.45×10^{5}
V	292-420	60.7	3.26×10^{5}	3.95×10^{4}
VI	421-442	18.9	1.87×10^{5}	1.12×10^{4}
VII	443-478	11.1	3.93×10^{4}	3.55×10^{3}
VIII	479-544	25.4	1.41×10^{6}	3.18×10^{4}

Only the residues in pools IV, VI and VIII have been processed to date.

Pool IV was chromatographed on a 36 g Celite column using system A (HBV = 40 ml) and one main radioactive peak was obtained in the 5th HBV. The material within this peak was combined to yield 5.0 mg of a residue containing 2.48×10^{6} dpm. This was chromatographed on a 1 g silica gel column to yield 3.6 mg of crystals having 1.84×10^{6} dpm ^{3}H and 4.63×10^{4}

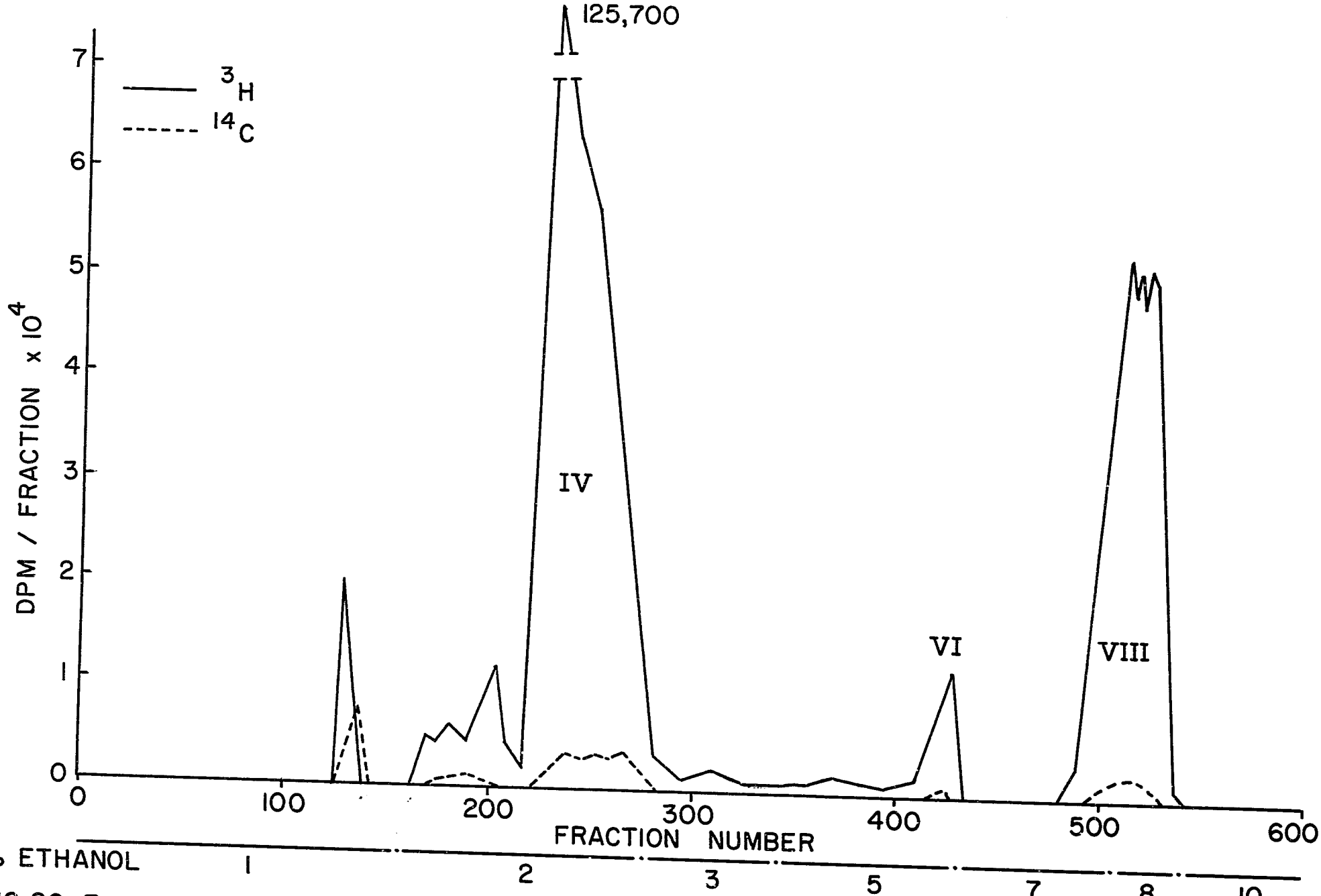

FIG. 20. Experiment 5. Silica gel chromatography. Sulfate fraction.

dpm ^{14}C. An infrared spectrum (KBr) indicated that these crystals were similar to 16α-hydroxydehydroisoandrosterone. Accordingly, an aliquot containing 4.55×10^4 dpm ^{3}H was mixed with 18.6 mg of carrier and the mixture prov to be radiochemically pure by crystallization as shown in Table 51. The

TABLE 51

Proof of Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated from the Sulfate Fraction of Pregnancy Urine

Crystallization	Crystals	Specific Activity (dpm ^{3}H/mg) $^{3}H/^{14}C$	Mother Liquor	$^{3}H/^{14}C$
16α-Hydroxydehydroisoandrosterone				
1	1370	49	4460	38
2	1000	66	1800	46
3	960	64	970	62
Calculated*	2450			
3β,16α-Diacetoxyandrost-5-en-17-one				
1	750	63	750	63
2	790	63	760	61
3	760	64	740	65
Calculated*	750			

*An aliquot of purified residue from pool IV containing 4.55×10^4 dpm 3H was mixed with 18.6 mg of carrier 16α-hydroxydehydroisoandrosterone and crystallized. The third crystals and mother liquor were acetylated and the product chromatographed on a small alumina column prior to crystallization. The calculated specific activity of the diacetate was based on the final specific activities of 16α-hydroxydehydroisoandrosterone and the altered molecular weight.

data in this table indicated that the original material was 45% pure. Another

aliquot was acetylated with ^{14}C-acetic anhydride solution "B". The product was mixed with carrier diacetate and the mixture was chromatographed on a small alumina column prior to crystallization, and again after the formation of 3β,16α-diacetoxyandrost-5-en-17β-ol as shown in Table 52. The specific

TABLE 52

Proof of Radiochemical Purity of 3β,16α-Diacetoxyandrost-5-en-17-one After Acetylation of an Aliquot of Purified Material from Pool IV with ^{14}C-Acetic Anhydride Solution "B"

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3β,16α-Diacetoxyandrost-5-en-17-one				
1	1750	20.3	5570	6.9
2	1700	20.0	2020	16.0
3	1720	20.5	1770	19.9
3β,16α-Diacetoxyandrost-5-en-17β-ol				
1	1720	19.7	1680	20.3
2	1710	21.3	1730	19.3
Calculated*	2420			

*An aliquot of purified material from pool IV containing 5.90×10^4 dpm ^3H was acetylated with ^{14}C-acetic anhydride solution "B" and the product was mixed with 17.2 mg of carrier 3β,16α-diacetoxyandrost-5-en-17-one. The mixture was chromatographed on a small alumina column to yield 15.1 mg of material containing 3.65×10^4 dpm ^3H. The third crystals and mother liquor were reduced with $NaBH_4$, and the product was chromatographed on a small alumina column prior to crystallization.

activity of 16α-hydroxydehydroisoandrosterone was calculated to be 1.12×10^6 dpm ^3H/mg, $^3H/^{14}C = 64$.

Chromatography of the residue of pool VI, Figure 20, Table 50, on 3 papers

in system Y for 12 hours yielded one main radioactive zone with the same mobility as estriol. The eluted material was chromatographed on a small silica gel column to yield 2.4 mg of residue containing 1.16×10^5 dpm ^3H and 7.66×10^4 dpm ^{14}C. It was crystallized from methanol to give 1.3 mg of fine needles with a specific activity of 4.84×10^4 dpm/mg. Its infrared spectrum (KBr) was identical to that of estriol. The remaining crystals were mixed with carrier estriol and proved to be radiochemically pure on crystallization as shown in Table 53.

TABLE 53

Proof of Radiochemical Purity of Estriol Isolated from the Sulfate Fraction of Pregnancy Urine

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
Estriol				
1	1700	12.7	2020	12.5
2	1670	12.5	1690	12.9
Calculated*	1700			
Estriol triacetate				
1	1270	13.0	1070	13.1
2	1130	12.8	1240	13.0
Calculated*	1170			

*A total of 3.52×10^4 dpm of ^3H from the crystals of pool VI was mixed with 19.6 mg of estriol carrier and crystallized. The second crystals and mother liquor were combined and acetylated with non-labelled acetic anhydride and the product was chromatographed on a small alumina column prior to crystallization. The calculated specific activity of the triacetate was based on the final specific activities of the estriol and the altered molecular weight.

The residue of pool VIII, Figure 20, Table 50, was chromatographed on

a small silica gel column and elution with 5-7% ethanol in methylene chloride yielded 10.3 mg of brown oil which contained 1.32×10^6 dpm ^3H and 3.22×10^4 dpm ^{14}C. Crystallization from methanol afforded 1.6 mg of fine needles: mp 264-167°C, specific activity 7.38×10^5 dpm ^3H/mg. Its infrared spectrum (KBr) was identical to that of Hirschmann's triol. A total of 7.78×10^4 dpm of the crystals was mixed with carrier Hirschmann's triol and crystallizaed to constant specific activity as shown in Table 54.

TABLE 54

Proof of Radiochemical Purity of Hirschmann's triol Isolated from the Sulfate Fraction of Pregnancy Urine

Crystallization	Specific Activity (dpm ^3H/mg)			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	5780	47	3930	48
2	5460	51	4630	48
3	5480	51	5390	54
4	5280	56	5070	53
Calculated*	5190			

*A total of 7.78×10^4 dpm of crystalline Hirschmann's triol from pool VIII was mixed with 15 mg carrier.

The combined glucosiduronidate fraction, 710 mg, was chromatographed on a 150 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described, and the elution of radioactivity is shown in Figure 21. The mode of elution, the weight and radioactivity of each pool are shown in Table 55.

Chromatography of pool IV on paper in system N for 29 hours and in

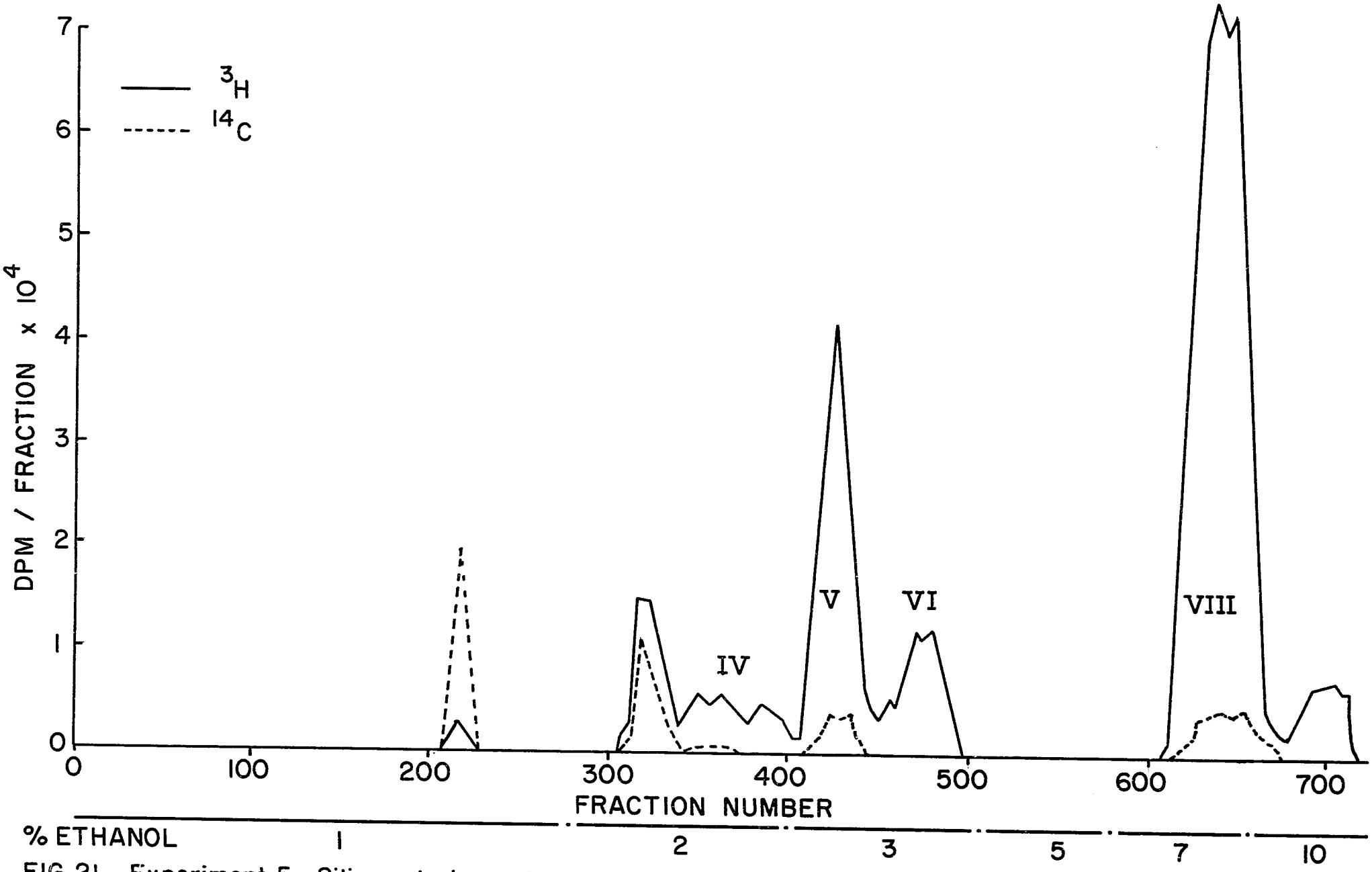

FIG. 21. Experiment 5. Silica gel chromatography. Glucosiduronidate fraction.

TABLE 55

Elution of Radioactive Material After Silica Gel Column Chromatography of the Glucosiduronidate Fraction of Pregnancy Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm) ^{3}H	^{14}C
I	216-240	48.0	7.67×10^{4}	1.13×10^{5}
II	241-312	19.3	1.35×10^{5}	7.47×10^{4}
III	313-347	19.8	2.59×10^{5}	7.28×10^{4}
IV	348-405	34.9	2.63×10^{5}	1.97×10^{4}
V	406-445	123.7	1.03×10^{6}	4.30×10^{4}
VI	446-495	42.9	4.01×10^{5}	4.11×10^{4}
VII	496-615	100.1	1.42×10^{5}	1.97×10^{4}
VIII	616-680	112.4	3.41×10^{6}	1.59×10^{5}
IX	681-730	38.3	3.95×10^{5}	3.62×10^{4}

system Q for 3 hours yielded one radioactive zone with the same mobility as 16α-hydroxyandrostenedione. The eluted material contained 8.27×10^{4} dpm ^{3}H and 2.30×10^{3} dpm ^{14}C. Using Siiteri's method (165) one half of the eluate was acetylated with non-labelled acetic anhydride and the other half with ^{14}C-acetic anhydride solution "A". The products were mixed with carrier acetate and chromatographed on alumina columns and crystallized as shown in Table 56. The specific activity of 16α-hydroxyandrostenedione was calculated to be 2.23×10^{5} dpm ^{3}H/mg; $^{3}H/^{14}C = 41.8$.

The residue of pool V, Figure 21, was chromatographed on a 120 g Celite column using system A (HBV = 170 ml) and one main radioactive peak was obtained in the 3rd and 4th HBV. The material within this peak was combined

TABLE 56

Proof of Radiochemical Purity of 16α-Hydroxyandrostenedione Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Specific Activity (dpm ^3H/mg)

Crystallization	½ of 16α-Hydroxyandrostenedione Acetylated with Non-labelled Acetic Anhydride				½ of 16α-Hydroxyandrostenedione Acetylated with ^{14}C-acetic anhydride Solution "A"			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	2160	51	3200	34	1560	1.7	1090	0.1
2	2070	45	2400	39	1530	1.5	1550	0.5
3	1950	43	2110	39	1530	1.6	1540	1.5
4	1990	42	2040	41				
Calculated	2040*				1500**			

*After acetylation with non-labelled acetic anhydride and chromatography of the product with carrier 16α-acetoxyandrostenedione on an alumina column, a total of 17.2 mg of colorless oil was obtained containing 3.51×10^4 dpm ^3H.

**Chromatography of the acetylated product with carrier 16α-acetoxyandrostenedione on an alumina column yielded 22.1 mg of colorless oil containing 3.32×10^4 dpm ^3H.

Corrected $^3H/^{14}C = 1.6$

Specific activity of 16α-hydroxyandrostenedione = 2.23×10^5 dpm ^3H/mg.

(35.2 mg, 7.76×10^5 dpm ^3H and 2.06×10^4 dpm ^{14}C) and chromatographed on 4 papers in system P for 18 hours. One main radioactive peak was obtained with a mobility of 20.9 cm, slightly less polar than 3α,16α-dihydroxyandrost-5-en-17-one. The eluate which contained 3.58×10^5 dpm ^3H and 1.05×10^4 dpm ^{14}C was chromatographed on paper in system Q for 7 hours to yield two radioactive areas at distances of 26.3 cm and 34.1 cm corresponding to 3α-16α-dihydroxyandrost-5-en-17-one and 16α-hydroxyandrosterone respectively. The more polar area was eluted to give 2.59×10^5 dpm ^3H and 6.70×10^3 dpm ^{14}C. One half was acetylated with non-labelled acetic anhydride and the other with ^{14}C-acetic anhydride solution "A" as previously described and the products were chromatographed to constant $^3H/^{14}C$ ratio and again after the addition of labelled carrier as shown in Table 57. From the data shown in this table,

TABLE 57

Proof of Radiochemical Purity of 3α,16α-Dihydroxyandrost-5-en-17-one Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Chromatography	Support	System	Rf or HBV	$^3H/^{14}C$ After Acetylation With ^{14}C-Acetic anhydride	$^3H/^{14}C$ After Acetylation With Non-labelled Acetic anhydride
1	Paper	W	0.74	1.3	40.2
2	Paper	W reversed phase	0.50	1.3	39.7
3	Celite	M	5	1.4	38.4
4	Paper	M	0.57	4.5	9.0
5	Celite	C	3,4	4.6	8.9
Calculated*				4.4	9.2

*After the third chromatographic step the acetate prepared from ^{14}C-acetic anhydride contained 4.96×10^3 dpm ^3H and 3.52×10^3 dpm ^{14}C. This was mixed with 1.05×10^4 dpm ^3H of 3α,16α-diacetoxyandrost-5-en-17-one to give a predicted $^3H/^{14}C$ ratio of 4.4 and the mixture was rechromatographed in systems 4 & 5. At the same stage the acetate prepared from non-labelled acetic anhydride (3.24×10^3 dpm ^3H and 84 dpm ^{14}C) was mixed with labelled carrier acetate (5.87×10^3 dpm ^3H and 9.06×10^2 dpm ^{14}C) to give a calculated $^3H/^{14}C$ of 9.2.

it was possible to assign the structure of 3α,16α-dihydroxyandrost-5-en-17-one to this metabolite. The specific activity of 3α,16α-dihydroxyandrost-5-en-17-one was calculated to be 4.34×10^5 dpm ^3H/mg, $^3H/^{14}C$ = 38.4.

From pool VI, Figure 21, Table 55, a residue was obtained which was chromatographed on paper in system Q for 7 hours and in system P for 22 hours to yield one radioactive zone slightly less polar than 16α-hydroxydehydroisoandrosterone. The eluate was chromatographed on a small silica gel column to yield 1.71×10^5 dpm ^3H and 4.40×10^3 dpm ^{14}C. One-half of the eluted material was acetylated with non-labelled acetic anhydride and the product was mixed with carrier 3β,16α-diacetoxyandrost-5-en-17-one. The mixture was chromatographed on a small alumina column prior to crystallization as shown in Table 58. The data in this table indicate that 16α-hydroxydehydroisoandrosterone was not present in pool VI.

TABLE 58

Purity of 3β,16α-Diacetoxyandrost-5-en-17-one Prepared from the Material in Pool VI

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	170	28.3	8960	34.6
2	20	10.6	1080	19.8
3	-	0	130	53
Calculated*	3510			

*A total of 14.2 mg of material containing 4.99×10^4 dpm ^3H was eluted after alumina column chromatography.

The residue of pool VIII, Figure 21, Table 55, was chromatographed on a

100 g Celite column in system K (HBV = 130 ml) and one main radioactive peak was obtained in the 3rd and 4th HBV. The material within this peak was combined to yield 97.9 mg of residue containing 3.30×10^6 dpm ^3H and 1.44×10^5 dpm ^{14}C. This was crystallized to constant specific activity and again after the formation of the triacetate as shown in Table 59. The infrared spectra (KBr) of the unconjugated and acetylated products were identical to those of estriol and estriol triacetate respectively.

TABLE 59

Proof of Radiochemical Purity of Estriol Isolated from the Glucosiduronidate Fraction of Pregnancy Urine

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
Estriol				
1	53,430	21.0	18.390	22.0
2	51,630	20.6	49,610	20.9
Estriol triacetate				
1	36,940	20.9	32,520	21.5
2	36,980	19.9	35,140	20.1
Calculated*	35,210			

*After Celite column chromatography the residue of pool VIII was crystallized twice. The calculated specific activity of the triacetate was based on the final specific activities of the estriol and the altered molecular weight.

EXPERIMENT 6. Metabolism of 16α-Hydroxydehydroisoandrosterone by the Normal Mal

A normal 23-year old male was injected intravenously with (7α-^{3}H)-16α-hydroxydehydroisoandrosterone (Lot #2, 2.40x107 dpm in 75 μg). Urine was collected for five days and the conjugates in the urine were hydrolysed to give a sulfate fraction and glucosiduronidate fraction as previously described. The conjugates from the fifth day were accidentally lost during hydrolysis. Accordingly, the extracts from the first four days were further processed and Table 60 shows the radioactivity in these extracts.

TABLE 60

Radioactivity in the Extracts of the Sulfate and Glucosiduronidate Fractions of Normal Male Urine

Day	Sulfates (dpm)	% of Injected Dose	Glucosiduronidates	% of Injected Dose
1	$4.42x10^{6}$	18.4	$1.16x10^{7}$	48.5
2	$4.92x10^{5}$	2.05	$5.90x10^{5}$	2.24
3	$6.20x10^{4}$	0.26	$2.10x10^{5}$	0.87
4	$2.27x10^{4}$	0.09	$1.08x10^{5}$	0.45
Total	$5.00x10^{6}$	20.80	$1.25x10^{7}$	51.06

The combined sulfate extracts, 602 mg, was chromatographed on a 150 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described and the distribution of radioactivity obtained is shown in Figure 22. The mode of elution, the weight, and radioactivity of

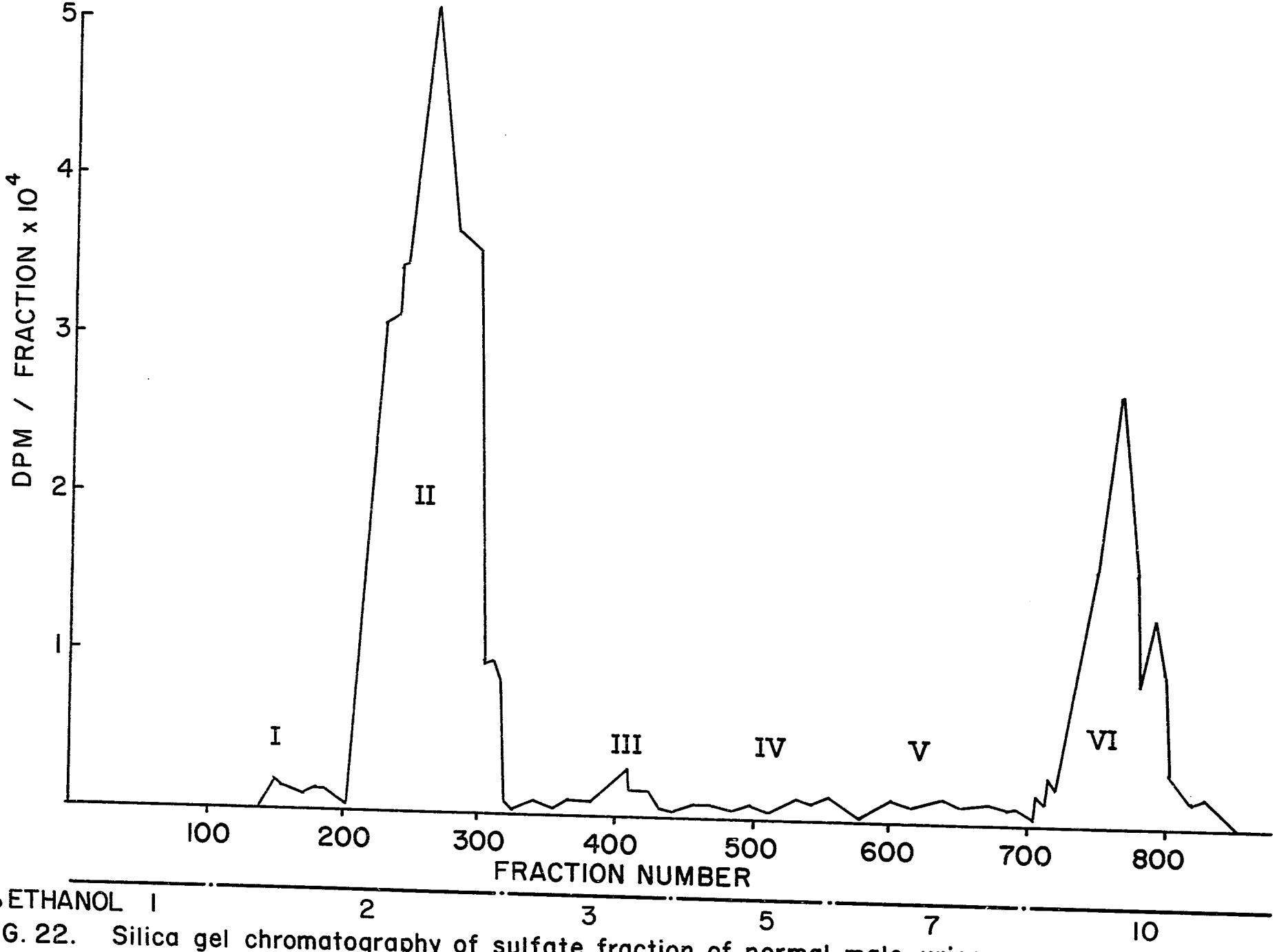

FIG. 22. Silica gel chromatography of sulfate fraction of normal male urine.

each pool are shown in Table 61.

TABLE 61

Elution of Radioactive Material after Silica Gel Column Chromatography of the Sulfate Fraction of Normal Male Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	124-204	60.1	1.42×10^5
II	205-331	42.4	2.38×10^6
III	332-460	22.2	2.06×10^6
IV	461-579	47.4	1.46×10^5
V	580-684	31.9	1.01×10^5
VI	685-840	37.6	2.23×10^5

Chromatography of the residue of pool II on a 42 g Celite column using system A accomplished no useful resolution. The combined residue after this procedure weighed 39.9 mg and contained 2.30×10^6 dpm. It was chromatographed on 4 papers in system Q for 7½ hours and three zones of radioactivity were observed at average mobilities of 17.1 cm, (IIA), 24.0 cm (IIB) and 29.3 cm (IIC) respectively. The residue of IIA (9.7 mg and 7.60×10^5 dpm) corresponding to 16α-hydroxydehydroisoandrosterone was chromatographed on a 1 g silica gel column to yield 4.6 mg of yellow oil containing 6.57×10^5 dpm. Attempts to crystallize this oil failed but the infrared spectrum (KBr) of an aliquot indicated that it contained a large percentage of 16α-hydroxydehydroisoandrosterone. An aliquot containing 1.50×10^5 dpm was acetylated with ^{14}C-acetic anhydride solution "B", mixed with carrier and crystallized. Constant specific activity was achieved after the third crystallization and again after $NaBH_4$ reduction

as shown in Table 62. The specific activity of 16α-hydroxydehydroisoandrosterone was calculated as 2.34×10^5 dpm/mg.

TABLE 62

Proof of Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated From the Sulfate Fraction of Normal Male Urine

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3β,16α-Diacetoxyandrost-5-en-17-one				
1	3240	4.3	8000	2.4
2	3230	4.2	3790	4.5
3	3070	4.2	3250	4.2
3β,16α-Diacetoxyandrost-5-en-17β-ol				
1	3080	4.0	2820	4.0
2	3050	4.0	2950	4.2
Calculated*	3270			

*An aliquot of IIA containing 1.50×10^5 dpm was acetylated with acetic anhydride solution "B" and the product was mixed with 26.4 mg of carrier 3β,16α-diacetoxyandrost-5-en-17-one. Chromatography of the mixture on a small alumina column yielded 22.5 mg of crystalline material containing 7.36×10^4 dpm ^3H and 2.21×10^4 dpm ^{14}C. These values were used to determine the calculated specific activity. The third crystals and mother liquor were used to prepare the 3β,16α-diacetoxyandrost-5-en-17β-ol.

The residue of IIB (5.4 mg and 3.02×10^5 dpm) corresponding to 3α,16α-dihydroxyandrost-5-en-17-one was chromatographed on paper in system Q for 7½ hours. It resolved into three radioactive peaks and the eluate of that corresponding to 3α,16α-dihydroxyandrost-5-en-17-one weighed 0.2 mg and contained 2.93×10^4 dpm. It was not possible to establish fully the identity of this metabolite because of the small amount of radioactivity recovered

after acetylation.

Eluate IIC corresponding in mobility to 16α-hydroxyandrosterone weighed 11.3 mg and contained 3.05×10^5 dpm. Chromatography on a 1 g silica gel column yielded 1.5 mg of yellow oil containing 2.69×10^5 dpm. This oil was acetylated with ^{14}C-acetic anhydride solution "A" and chromatographed to constant $^3H/^{14}C$ ratio using the Isotope Ratio Procedure as shown in Table 63. A constant $^3H/^{14}C$ ratio was achieved after the 4th chromatographic step

TABLE 63

Proof of Radiochemical Purity of 16α-Hydroxyandrosterone Isolated from the Sulfate Fraction of Normal Male Urine

Chromatography	Support	System	R_f or HBV	$^3H/^{14}C$
1	Paper	M	0.62	2.8
2	Paper reversed phase	W	0.63	2.9
3	Celite	M	4	3.7
4	Celite	C	3	3.9
5	Paper	M	0.64	6.7
6	Celite	C	2,3	6.4
Calculated*				6.3

*After the fourth chromatographic step the residue contained 4.59×10^4 dpm ^3H and 1.62×10^4 dpm ^{14}C. To this was added 3α,16α-diacetoxy-5α-androstan-17-one containing 5.61×10^4 dpm ^3H to give a predicted $^3H/^{14}C$ ratio of 6.3 and the mixture was rechromatographed in systems 5 and 6.

and again after the addition of carrier as shown in systems 5 and 6. From the data in Table 63, it was possible to assign the structure of 16α-hydroxyandrosterone to the metabolite in IIC. The specific activity of this metabolite was calculated to be 1.11×10^6 dpm/mg.

From pool III, Figure 22, Table 61, a residue was obtained which was chromatographed on 2 papers in system Q for 8 hours. One main radioactive peak was obtained at an average distance of 20.3 cm corresponding in mobility to 16α-hydroxydehydroisoandrosterone. The eluate was not processed further.

Pool VI, Figure 22, Table 61, yielded a residue which was chromatographed on 2 papers in system O for 24 hours and in system Y for 12 hours. All the radioactivity remained at the starting line after both procedures.

The combined glucosiduronidate fraction, 567 mg, was chromatographed on a 140 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described and the distribution of radioactivity obtained is shown in Figure 23. The mode of elution, the weight and radioactivity of each pool are shown in Table 64.

TABLE 64

Elution of Radioactive Material after Chromatography of the Glucosiduronidate Fraction of Normal Male Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	106-170	69.5	6.43×10^5
II	171-234	54.7	3.64×10^5
III	235-336	47.9	8.18×10^6
IV	337-444	51.2	1.45×10^6
V	445-507	19.1	5.56×10^5
VI	508-580	68.2	2.68×10^5
VII	581-669	38.6	6.99×10^5

Pools I, V, VI and VII contained material which was not characterized.

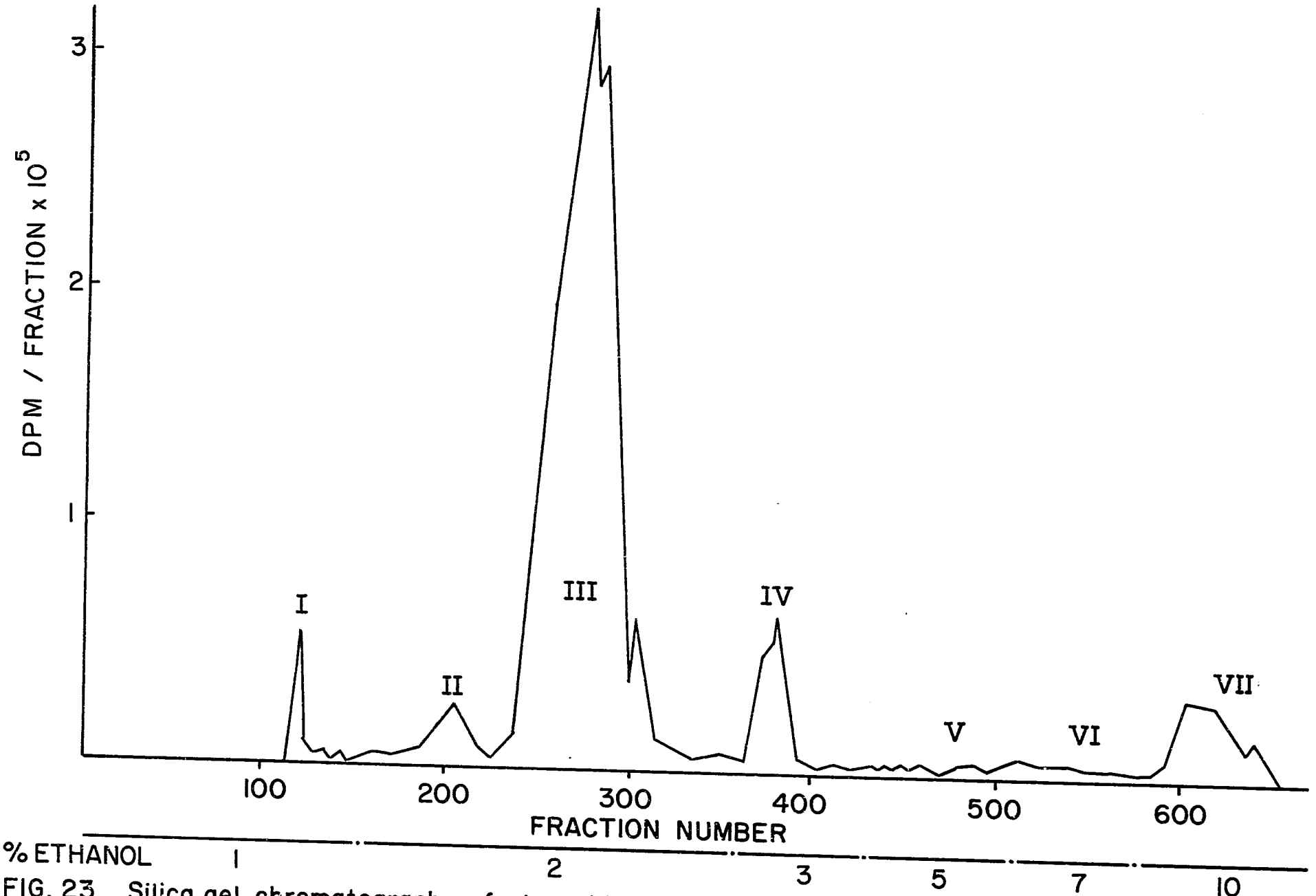

FIG. 23. Silica gel chromatography of glucosiduronidate fraction of normal male urine.

The residue of pool II was successively chromatographed on four thin layer plates in system ethyl acetate:n-hexane (3:1) and on paper in system Q for 3 hours to yield a zone of radioactivity with the same mobility as 16α-hydroxy-androstenedione. The eluted material was lost during the acetylation procedure.

Further purification of pool III, Figure 23, Table 64, on a 55 g Celite column using system A afforded no useful resolution of radioactivity and the material in the 3rd to 6th HBV was pooled to give 16.5 mg of yellow oil having 6.24×10^6 dpm. Chromatography on 4 papers in system Q for 7½ hours afforded three radioactive zones, IIIA corresponding to 16α-hydroxydehydroiso-androsterone, IIIB corresponding to 3α,16α-dihydroxyandrost-5-en-17-one, and IIIC corresponding to 16α-hydroxyandrosterone.

Eluate IIIA (6.8 mg and 3.19×10^5 dpm) was chromatographed on a small silica gel column to yield 0.9 mg of colorless oil having 2.20×10^5 dpm. This oil was acetylated with acetic anhydride solution "B", mixed with carrier 3β,16α-diacetoxyandrost-5-en-17-one, and crystallized, but constant specific activity could not be achieved as shown in Table 65.

TABLE 65

Purity of 3β,16α-Diacetoxyandrost-5-en-17-one Prepared from the Material in Eluate IIIA of the Glucosiduronidate Fraction of Normal Male Urine

Crystal-lization	Specific Activity (dpm ^3H/mg)			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	140	12.9	530	1.2
2	130	56.4	270	19.3
3	110	∞	180	8.3
Calculated*	190			

*The eluate of IIIA was acetylated with ^{14}C-acetic anhydride solution "B" and the product mixed with 32.4 mg carrier 3β,16α-diacetoxyandrost-5-en-17-one. The mixture was chromatographed on a small alumina column to yield 31.0 mg of material containing 5.88×10^3 dpm ^3H. These values were used to determine the calculated specific activity.

Chromatography of the eluate of IIIB (8.1 mg and 3.37×10^6 dpm) on a small silica column afforded 1.7 mg of yellow oil which contained 2.34×10^6 dpm. An aliquot, 3.50×10^5 dpm, was acetylated with acetic anhydride solution "A" and chromatographed to constant $^3H/^{14}C$ ratio and again after the addition of carrier using the Isotope Ratio Procedure as shown in Table 66. From the

TABLE 66

Proof of Radiochemical Purity of 3α,16α-Dihydroxyandrost-5-en-17-one Isolated from the Glucosiduronidate Fraction of Normal Male Urine

Chromatography	Support	System	R_f or HBV	$^3H/^{14}C$
1	Paper	M	0.54	21.6
2	Paper	W	0.75	23.9
3	Celite	M	4,5	29.4
4	Celite	C	3	30.0
5	Paper	M	0.56	11.2
6	Celite	C	3,4	11.4
Calculated*				10.9

*After the fourth chromatographic step the residue contained 7.27×10^4 dpm ^3H and 2.63×10^3 dpm ^{14}C. This was mixed with 3α,16α-diacetoxyandrost-5-en-17-one containing 2.31×10^4 dpm ^3H and 5.17×10^3 dpm ^{14}C to give a predicted ratio of 10.9 and the mixture was rechromatographed in systems 5 and 6.

data in this table it was possible to assign the structure of 3α,16α-dihydroxyandrost-5-en-17-one to the metabolite in IIIB. The specific activity of this metabolite was calculated to be 8.52×10^6 dpm/mg.

From IIIC an eluate was obtained which weighed 7.8 mg and contained 1.49×10^6 dpm. It was chromatographed on a 1 g silica gel column to yield 3.5 mg of yellow

oil having 1.26×10^6 dpm. An aliquot, 5.05×10^5 dpm, was acetylated with ^{14}C-acetic anhydride solution "A" and chromatographed to constant $^3H/^{14}C$ ratio and again after addition of carrier as shown in Table 67. From the data in

TABLE 67

Proof of Radiochemical Purity of 16α-Hydroxyandrosterone Isolated from the Glucosiduronidate Fraction of Normal Male Urine

Chromatography	Support	System	R_f or HBV	$^3H/^{14}C$
1	Paper	M	0.64	17.0
2	Paper reversed phase	W	0.71	14.4
3	Celite	M	4	30.5
4	Celite	C	2,3	30.3
5	Paper	M	0.62	38.8
6	Celite	C	2,3	37.5
Calculated*				37.8

*After the fourth chromatographic step the residue contained 6.68×10^4 dpm ^3H and 2.21×10^3 dpm ^{14}C. To this residue was added 3α,16α-diacetoxy-5α-androstan-17-one containing 1.66×10^4 dpm ^3H to give a predicted $^3H/^{14}C$ ratio of 37.8 and the mixture was rechromatographed in systems 5 and 6.

this table it was possible to assign the structure of 16α-hydroxyandrosterone to the metabolite in pool IIIC. The specific activity of this metabolite was calculated as 8.57×10^6 dpm/mg.

The residue of pool IV, Figure 23, Table 64, was chromatographed on a 50 g Celite column using system A (HBV = 70 ml) and one main radiactive peak was obtained in the 3rd and 4th HBV. The material within this peak was pooled to yield 10.2 mg of yellow oil containing 1.24×10^6 dpm. An aliquot, 4.82×10^5 dpm, was acetylated with ^{14}C-acetic anhydride solution "A" and chromatographed

to constant $^{3}H/^{14}C$ ratio using the Isotope Ratio Procedure, as shown in Table 68. From the data in this table it was possible to assign the structure of 16α-hydroxyetiocholanolone to the metabolite in pool IV. The specific activity of this metabolite was calculated to be 3.15×10^{6} dpm/mg.

TABLE 68

Proof of Radiochemical Purity of 16α-Hydroxyetiocholanolone Isolated from the Glucosiduronidate Fraction of Normal Male Urine

Chromatography	Support	System	R_f of HBV	$^{3}H/^{14}C$
1	Paper	M	0.69	4.5
2	Paper reversed phase	W	0.66	4.6
3	Celite	M	3,4	10.5
4	Celite	C	3	11.2
5	Paper	M	0.71	15.2
6	Celite	C	2,3	15.5
Calculated*				15.7

*After the fourth chromatographic step the residue contained 5.55×10^{4} dpm ^{3}H and 4.95×10^{3} dpm ^{14}C. To this residue was added 3α,16α-diacetoxy-5β-androstan-17-one containing 2.19×10^{4} dpm ^{3}H to give a predicted $^{3}H/^{14}C$ ratio of 15.7 and the mixture was rechromatographed in systems 5 and 6.

EXPERIMENT 7. Metabolism of 16α-Hydroxydehydroisoandrosterone by the Normal Female.

A normal 23-year old subject in the luteal phase of the menstrual cycle was injected intravenously with 2.34×10^7 dpm, 71 μg, of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone (Lot #2). Urine was collected for five days and hydrolysed to give a sulfate fraction and a glucosiduronidate fraction as previously described. Table 69 shows the radioactivity in the urine and extracts of each day.

The combined sulfate fraction (1.2 g) was chromatographed on a 200 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described and the distribution of radioactivity obtained is shown in Figure 24. The mode of elution, the weight and radioactivity of each pool are shown in Table 70.

TABLE 70

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

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	167-200	21.9	9.67×10^4
II	337-424	128.5	1.51×10^6
III	425-478	16.1	3.55×10^5
IV	479-620	93.4	2.26×10^5
V	621-772	114.5	1.31×10^5
VI	773-860	41.8	6.61×10^5

Pool II contained a residue which was chromatographed on a 120 g Celite column using system A and a plot of radioactivity versus fraction number is

TABLE 69

Radioactivity in the Urine and Conjugates Excreted as Sulfates and Glucosiduronidates After the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone to a Normal Female

Day	Crude Urine	% of injected dose	Sulfates	% of injected dose	Glucosiduronidates	% of injected dose
1	1.54×10^7	66.01	2.76×10^6	11.78	1.33×10^7	56.70
2	7.78×10^5	3.32	1.76×10^5	1.75	5.34×10^5	2.28
3	4.23×10^5	1.80	1.56×10^5	0.66	3.47×10^5	1.48
4	2.28×10^5	0.97	1.12×10^5	0.47	1.02×10^5	0.43
5	2.02×10^5	0.85	1.02×10^5	0.43	6.00×10^4	0.25
Total	1.71×10^7	72.95	3.30×10^6	14.09	1.43×10^7	61.14

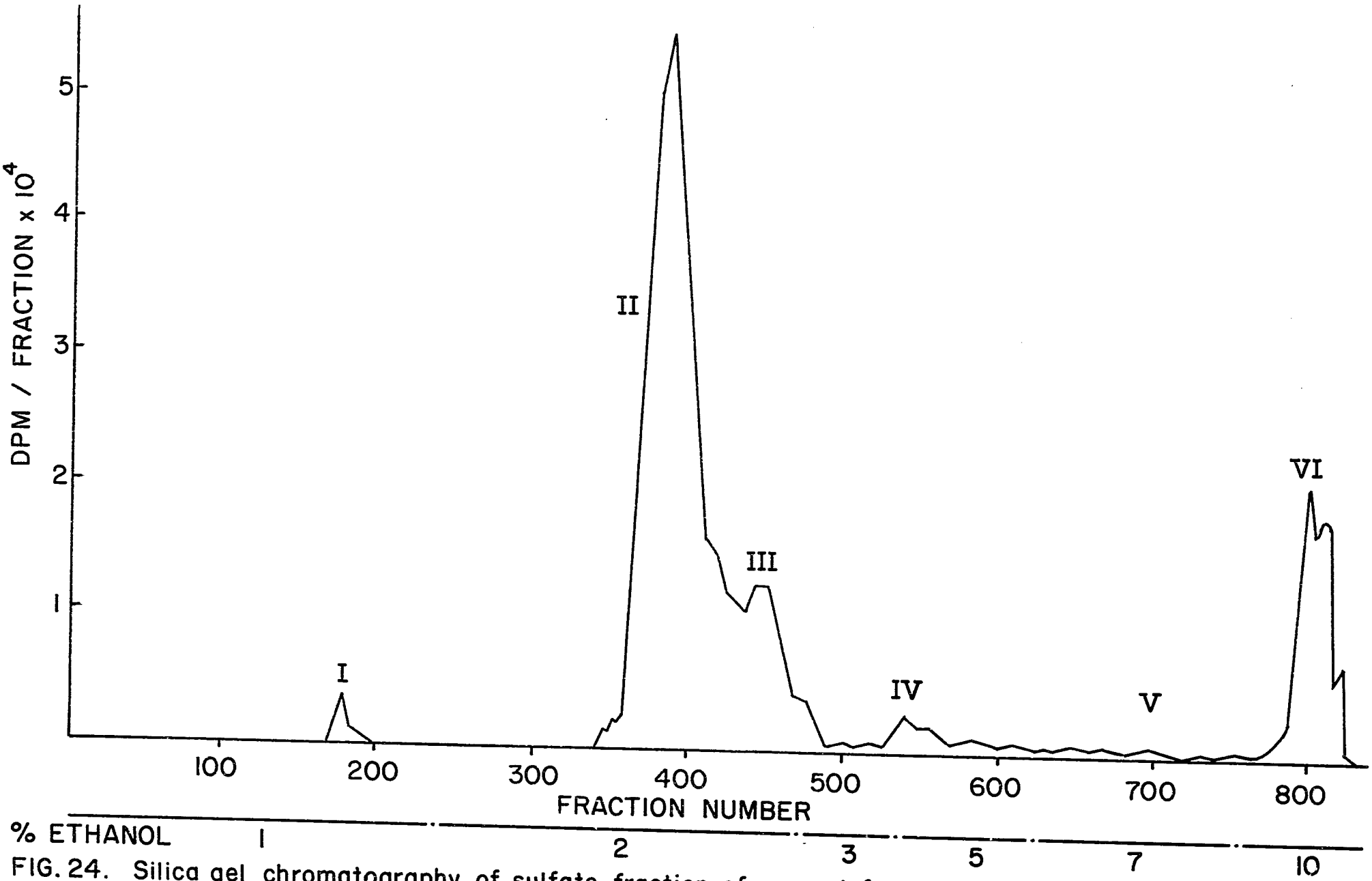

FIG. 24. Silica gel chromatography of sulfate fraction of normal female urine.

shown in Figure 25. Fractions 36-48, 49-68 and 69-87 constituted pools IIA, IIB and IIC respectively. Pool IIA (4.4 mg and 1.49×10^5 dpm) was chromatographed on one paper in system Q for 8 hours to yield one radioactive zone corresponding in mobility to 16α-hydroxyandrosterone. The eluate (1.8 mg and 1.30×10^5 dpm) was acetylated with ^{14}C-acetic anhydride solution "A" and chromatographed using the Isotope Ratio Procedure. Constant $^3H/^{14}C$ ratio was achieved after the fourth chromatographic procedure and again after the addition of carrier as shown in Table 71. From the data in this table it was

TABLE 71

Proof of Radiochemical Purity of 16α-Hydroxyandrosterone Isolated From the Sulfate Fraction of Normal Female Urine

Chromatography	Support	System	R_f or HBV	$^3H/^{14}C$
1	Paper	M	0.60	1.2
2	Paper	W	0.86	1.2
3	Celite	M	4,5	1.9
4	Celite	C	3	2.0
5	Paper	M	0.63	5.3
6	Celite	C	3	5.2
Calculated*				5.6

*After the fourth chromatographic step the residue contained 8.59×10^3 dpm ^3H and 4.36×10^3 dpm ^{14}C. To this residue was added 3α,16α-diacetoxy-5α-androstan-17-one containing 1.58×10^4 dpm ^3H to give a predicted $^3H/^{14}C$ ratio of 5.6 and the mixture was rechromatographed in systems 5 and 6.

possible to assign the structure of 16α-hydroxyandrosterone to the metabolite in pool IIA. The specific activity of this metabolite was calculated to be

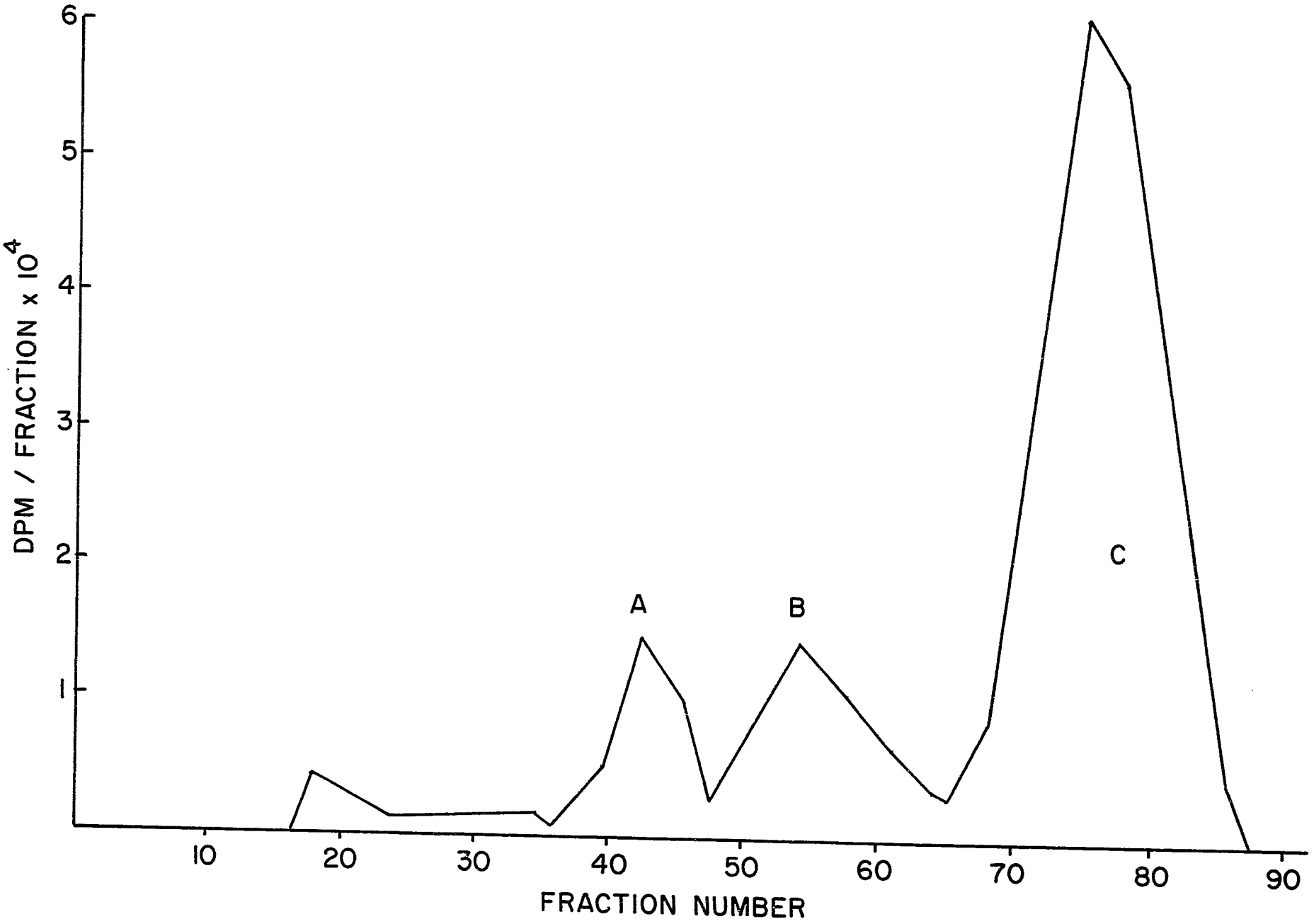

FIG. 25. Celite column chromatography of residue from pool II, Fig. 24, using System A.

$6.63x10^5$ dpm/mg.

Chromatography of pool IIB (5.5 mg and $2.41x10^5$ dpm) on one paper in system Q for 8 hours yielded two radioactive zones, BI and BII, corresponding in mobilities to 16α-hydroxydehydroisoandrosterone and 3α,16α-dihydroxyandrost-5-en-17-one respectively. Elution of the material from zone BII afforded 1.9 mg of colorless oil having $1.20x10^5$ dpm. This oil was acetylated with ^{14}C-acetic anhydride solution "B" and chromatographed as shown in Table 72. It

TABLE 72

Purity of 3α,16α-Dihydroxyandrost-5-en-17-one Isolated from the Sulfate Fraction of Normal Female Urine

Chromatography	Support	System	R_f of HBV	$^3H/^{14}C$
1	Paper	M	0.66	7.1
2	Paper reversed phase	W	0.61	7.3
3	Celite	M	5,6	6.9

*After the third chromatographic step there was insufficient radioactivity for further processing.

was not possible to fully establish the identity of this metabolite. The specific activity of the metabolite at this stage was calculated to be 2.94 $x10^5$ dpm/mg.

Residue IIC (12.1 mg and $8.69x10^5$ dpm) was chromatographed on a small silica gel column to yield 5.2 mg of yellow oil containing $7.00x10^5$ dpm. Crystallization from acetone-Skellysolve B afforded 2.0 mg of crystals: mp 176-179°C, mmp 175-179°C; infrared spectrum (KBr) was identical to that of 16α-hydroxydehydroisoandrosterone. An aliquot containing $7.04x10^4$ dpm was acetylated with acetic anhydride solution "B", mixed with 24.3 mg carrier diacetate, and the mixture was chromatographed on a small alumina column prior

to crystallization. Constant specific activity was achieved after the third crystallization and again after $NaBH_4$ reduction as shown in Table 73.

TABLE 73

Proof of Radiochemical Purity of 3β,16α-Diacetoxyandrost-5-en-17-one Prepared from Pool IIC of the Sulfate Fraction of Normal Female Urine

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3β,16α-Diacetoxyandrost-5-en-17-one				
1	2010	3.1	2260	1.3
2	2480	3.1	2250	2.5
3	2190	2.9	2190	3.1
3β,16α-Diacetoxyandrost-5-en-17β-ol				
1	2060	3.0	1930	3.0
2	2070	2.9	2140	3.1
Calculated*	2240			

*An aliquot of purified IIC was acetylated with acetic anhydride solution "B" and the product was mixed with 24.3 mg of carrier 3β,16α-diacetoxyandrost-5-en-17-one. Chromatography of the mixture on a small alumina column yielded 18.6 mg of crystalline residue containing 4.16×10^4 dpm ^3H and 1.47×10^4 dpm ^{14}C. These values were used to obtain the calculated specific activity. The third crystals and mother liquor were used to prepare the 3β,16α-diacetoxyandrost-5-en-17β-ol.

The specific activity of 16α-hydroxydehydroisoandrosterone was calculated to be 1.69×10^5 dpm/mg.

Further purification of the residue of pool III, Figure 24, Table 70, was accomplished by chromatography on two papers in system Q for 8 hours. One symmetrical zone of radioactivity corresponding in mobility to 16α-hydroxyetiocholanolone (6.9 mg and 2.77×10^5 dpm) was eluted and chromatographed on a small silica gel column to yield 0.9 mg of colorless oil containing 1.87×10^5

dpm. This oil was acetylated with ^{14}C-acetic anhydride solution "A" and chromatographed to constant $^{3}H/^{14}C$ ratio using the Isotope Ratio Procedure as shown in Table 74. From the data in this table it was possible to assign

TABLE 74

Proof of Radiochemical Purity of 16α-Hydroxyetiocholanolone Isolated from The Sulfate Fraction of Normal Female Urine

Chromatography	Support	System	R_f or HBV	$^{3}H/^{14}C$
1	Paper	M	0.71	2.0
2	Paper	W	0.82	2.2
3	Celite	M	4,5	2.3
4	Paper	M	0.70	5.5
5	Celite	C	2,3	5.3
Calculated*				5.6

*After the third chromatographic step the residue contained 7.62×10^3 dpm ^{3}H and 3.16×10^3 dpm ^{14}C. To this residue was added 3α,16α-diacetoxy-5β-androstan-17-one containing 1.01×10^4 dpm ^{3}H to give a predicted $^{3}H/^{14}C$ ratio of 5.6 and the mixture was rechromatographed in systems 4 and 5.

the structure of 16α-hydroxyetiocholanolone to the metabolite in pool III. The specific activity of this metabolite was calculated to be 5.58×10^5 dpm/mg.

The residue of pool VI, Figure 24, Table 70, was chromatographed successively on paper in system Y for 12 hours and in system O for 37 hours but the radioactivity remained at the starting line. This material was not processed further.

The combined glucosiduronidate fraction, 1.2 g , was chromatographed on a 300 g silica gel column using increasing concentrations of ethanol in methylene chloride as previously described. In Figure 26 is shown the dis-

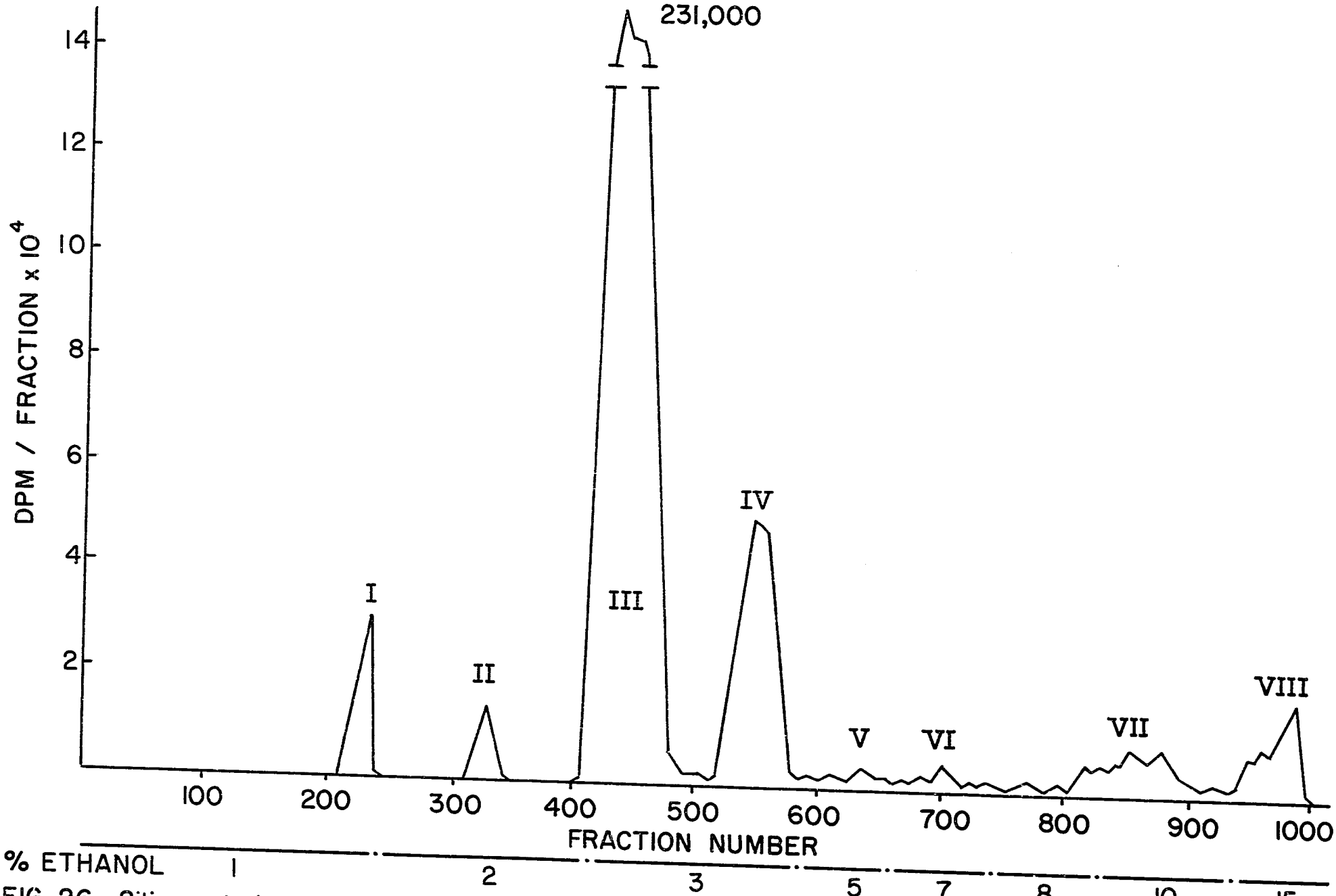

FIG. 26. Silica gel chromatography of glucosiduronidate fraction of normal female urine.

tribution of radioactivity. The mode of elution, the weight and radioactivity of each pool are shown in Table 75. Pools I, V, VI and VIII contained material which was not identified.

TABLE 75

Elution of Radioactive Material after Silica Gel Column Chromatography of the Glucosiduronidate Fraction of Normal Female Urine

Pool	Fraction No.	Weight (mg)	Radioactivity (dpm)
I	219-260	86.9	3.74×10^5
II	261-368	103.9	3.68×10^5
III	369-506	121.0	9.31×10^6
IV	507-585	42.3	1.91×10^6
V	586-655	61.5	1.96×10^5
VI	656-795	189.8	4.30×10^5
VII	796-910	67.2	3.71×10^5
VIII	911-1020	77.5	6.79×10^5

Chromatography of the residue of pool II on six thin layer plates in system n-hexane:ethyl acetate (1:2) yielded an area corresponding in mobility to 16α-hydroxyandrostenedione. The eluate from the plates was accidentally lost.

From pool III a residue was obtained which was chromatographed on a 120 g Celite column using system A and the distribution of radioactivity obtained is shown in Figure 27. Fractions 30-42, 43-60, 61-84 were pooled to give residues IIIA, IIIB and IIIC respectively. The residue of pool IIIA (20.9 mg and 2.18×10^6 dpm) was chromatographed on 4 papers in system Q for 7 hours to yield one main radioactive zone corresponding in mobility to 16α-hydroxyandrosterone. Elution from the papers yielded 18.8 mg of colorless

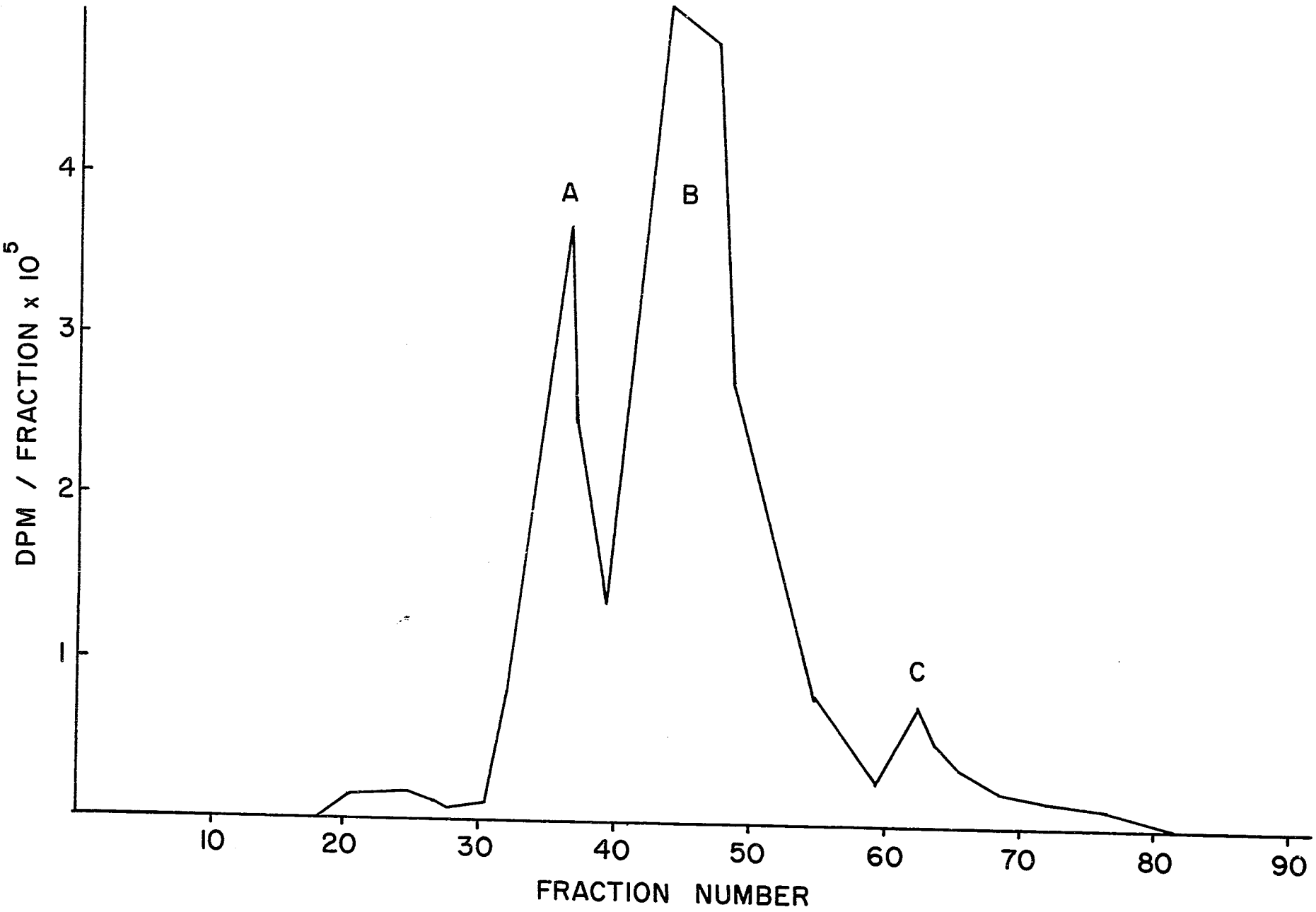

FIG. 27. Celite column chromatography of residue from pool III, Fig. 26, using System A.

oil containing 1.32×10^6 dpm. Chromatography on 2 papers in system P for 24 hours gave one radioactive peak at 40.0 cm on one paper while the radioactivity ran off the other paper. The eluate from the first paper (12.7 mg, 4.22×10^5 dpm) was chromatographed on a small silica gel column to yield 1.3 mg of residue containing 3.94×10^5 dpm. This residue was acetylated with ^{14}C-acetic anhydride solution "A" and the product chromatographed to constant $^3H/^{14}C$ ratio using the Isotope Ratio Procedure. Constant $^3H/^{14}C$ ratio was achieved after the fourth chromatographic step and again after the addition of labelled carrier as shown in Table 76. From the data in this table it was possible to

TABLE 76

Proof of Radiochemical Purity of 16α-Hydroxyandrosterone Isolated from the Glucosiduronidate Fraction of Normal Female Urine

Chromatography	Support	System	Rf or HBV	$^3H/^{14}C$
1	Paper	M	0.60	14.9
2	Paper	W	0.82	18.6
3	Celite	M	3,4	19.7
4	Celite	C	2,3	18.9
5	Paper	M	0.62	26.3
6	Celite	C	2,3	26.0
Calculated*				25.6

*After the fourth chromatographic step the residue contained 8.23×10^3 dpm ^3H and 4.35×10^2 dpm ^{14}C. To this was added 3α,16α-diacetoxy-5α-androstan-17-one containing 2.94×10^3 dpm ^3H to give a predicted $^3H/^{14}C$ ratio of 25.6 and the mixture was rechromatographed in systems 5 and 6.

assign the structure of 16α-hydroxyandrosterone to the metabolite in pool IIIA.

The specific activity of this metabolite was calculated to be 5.56×10^5 dpm/mg.

Residue IIIB, Figure 27, weighed 14.6 mg and contained 4.00×10^6 dpm. Chromatography on 2 papers in system Q for 8 hours yielded two radioactive zones BI and BII, corresponding in mobility to 16α-hydroxydehydroisoandrosterone, and 3α,16α-dihydroxyandrost-5-en-17-one respectively. The eluate of BII (13.9 mg and 3.01×10^6 dpm) was chromatographed on one paper in system P for 24 hours. One radioactive zone corresponding in mobility to 3α,16α-dihydroxyandrost-5-en-17-one was eluted to yield 5.1 mg of material containing 2.00×10^6 dpm. An aliquot containing 2.82×10^5 dpm was acetylated with ^{14}C-acetic anhydride solution "A" and the product chromatographed using the Isotope Ratio Procedure. Constant $^3H/^{14}C$ ratio was obtained after the third chromatographic step and again after the addition of labelled carrier as shown in Table 77.

TABLE 77

Proof of Radiochemical Purity of 3α,16α-Dihydroxyandrost-5-en-17-one Isolated from the Glucosiduronidate Fraction of Normal Female Urine

Chromatography	Support	System	R_f or HBV	$^3H/^{14}C$
1	Paper	M	0.60	19.3
2	Paper reversed phase	W	0.64	20.3
3	Celite	M	4,5	21.1
4	Celite	M	4,5	11.6
5	Paper	W	0.78	12.2
Calculated*				11.3

*After the third chromatographic step the residue contained 7.06×10^4 dpm ^3H and 3.16×10^3 dpm ^{14}C. To this was added 3α,16α-diacetoxyandrost-5-en-17-one containing 2.31×10^4 dpm ^3H and 5.17×10^3 dpm ^{14}C to allow for greater sensitivity in counting. This gave a predicted $^3H/^{14}C$ ratio of 11.3 and the mixture was rechromatographed in systems 4 and 5.

The specific activity of this metabolite (3α,16α-dihydroxyandrost-5-en-17-one) was calculated to be 5.76×10^6 dpm/mg.

Residue IIIC, Figure 27, 4.5 mg and 3.49×10^5 dpm, was chromatographed on paper in system Q for 8 hours. Two radioactive zones were obtained, CI and CII, with mobilities corresponding to 16α-hydroxydehydroisoandrosterone and 3α,16α-dihydroxyandrost-5-en-17-one respectively. The eluate of CI was mixed with that of BI (page 152) to yield 6.3 mg of residue containing 3.62×10^5 dpm. Chromatography of this mixture on one paper in system P for 24 hours gave one radioactive zone with the mobility of 16α-hydroxydehydroisoandrosterone. The eluate (7.4 mg and 1.36×10^5 dpm) was chromatographed on a small silica gel column to give 4.6 mg of yellow oil containing 8.64×10^4 dpm. This oil was acetylated with ^{14}C-acetic anhydride solution "A" and the product shown to be radiochemically pure by crystallization as shown in Table 78. From the data in this table the specific activity of 16α-hydroxydehydroisoandrosterone was calculated to be 5.80×10^6 dpm/mg.

Chromatography of the residue of pool IV, Figure 26, Table 75, on 5 papers in system R for 7 hours afforded one radioactive zone corresponding in mobility to 16α-hydroxyetiocholanolone. The eluted material (14.6 mg and 1.27×10^6 dpm) was chromatographed on a small silica gel column to give 4.5 mg of yellow oil containing 9.00×10^5 dpm. One-half of this eluate was acetylated with ^{14}C-acetic anhydride solution "A" and the product chromatographed using the Isotope Ratio Procedure previously described. Constant $^3H/^{14}C$ ratio was achieved after the fourth chromatographic procedure and again after the addition of labelled carrier as shown in Table 79. From the data in this table it was possible to assign the structure of 16α-hydroxyetiocholanolone to the metabolite in pool IV. The specific activity of this metabolite was calculated to be 1.56×10^6 dpm/mg.

TABLE 78

Proof of Radiochemical Purity of 3β,16α-Diacetoxyandrost-5-en-17-one Prepared from the Material in CI of the Glucosiduronidate Fraction of Normal Female Urine

Crystallization	Crystals	Specific Activity (dpm ^3H/mg) $^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
3β,16α-Diacetoxyandrost-5-en-17-one				
1	740	2.7	1700	0.1
2	700	15	810	0.5
3	750	20	730	3.6
4	730	21	710	10.0
5	730	22	700	21
3β,16α-Diacetoxyandrost-5-en-17β-ol				
1	690	20	700	21
2	710	21	680	20
Calculated*	1040			

*The residue of eluate CI was acetylated with acetic anhydride solution "A" and the product mixed with carrier 3β,16α-diacetoxyandrost-5-en-17-one before chromatography on a small alumina column. A total of 20.1 mg of crystalline material was eluted and it contained 2.09×10^4 dpm ^3H. The fifth crystals and mother liquor were used to prepare the 3β,16α-diacetoxyandrost-5-en-17β-ol.

TABLE 79

Proof of Radiochemical Purity of 16α-Hydroxyetiocholanolone Isolated from the Glucosiduronidate Fraction of Normal Female Urine

Chromatography	Support	System	R_f or HBV	$^3H/^{14}C$
1	Paper	M	0.68	5.2
2	Paper reversed phase	W	0.67	3.6
3	Celite	M	3	5.1
4	Celite	C	2,3	5.5
5	Paper	M	0.69	8.6
6	Celite	C	3	8.8
Calculated*				8.2

*After the fourth chromatographic step the residue contained 1.97×10^4 dpm ^3H and 3.52×10^3 dpm ^{14}C. To this residue was added 3α,16α-diacetoxy-5β-androstan-17-one containing 9.30×10^3 dpm ^3H to give a predicted $^3H/^{14}C$ ratio of 8.2 and the mixture was rechromatographed in systems 5 and 6.

From pool VII, Figure 26, Table 75, a residue was obtained which was chromatographed on a 40 g Celite column using system K (HBV = 50 ml). One main radioactive peak was observed in the 4th HBV and the material within this peak was pooled to give 5.6 mg of residue containing 1.72×10^5 dpm. Chromatography of this residue on paper in system Y for 12 hours afforded one radioactive zone with the mobility of estriol. The eluted material (3.7 mg and 1.49×10^5 dpm) was further purified by chromatography on a small silica gel column. Elution with 8% ethanol in methylene chloride afforded 2.1 mg of an oily residue containing 1.03×10^5 dpm. It was acetylated with ^{14}C-acetic anhydride solution "C" and the product was mixed with 21.6 mg of carrier triacetate. Chromatography on a small alumina column and crystallization of the product as shown in Table 80 indicated that the metabolite in pool VII was not estriol.

TABLE 80

Purity of Estriol Isolated from the Glucosiduronidate Fraction of Normal Female Urine

Crystallization	Specific Activity (dpm ^3H/mg)			
	Crystals	$^3H/^{14}C$	Mother Liquor	$^3H/^{14}C$
1	260	14.8	7260	18.5
2	80	43.3	4070	18.2
Calculated*	2710			

*After alumina column chromatography of the acetylated material from pool VII with 21.6 mg of carrier estriol triacetate, a total of 19.6 mg of oily residue was obtained and it contained 5.32×10^4 dpm ^3H.

DISCUSSION

In planning these studies it was immediately evident that a facile method would have to be developed for the preparation of labelled 16α-hydroxysteroids to be used as precursors. The recent review of Dorfman and Ungar (166) on the microbiological transformation of a number of steroids shows that various species of Streptomyces are capable of introducing a 16α-hydroxyl group on the preformed steroid nucleus. Hence, Streptomyces roseochromogenus, ATCC 3347, generously provided by Dr. C. Vezina, Ayerst Laboratories, was chosen to effect the conversion of labelled precursors to the 16α-hydroxylated products. Labelled 16α-hydroxydehydroisoandrosterone and 16α-hydroxyandrostenedione were thus prepared in good yield and with a high degree of purity. During the course of these studies it was found that dehydroisoandrosterone-3-sulfate could also be hydroxylated at the 16α-position thus providing a method for the preparation of this valuable precursor. This conversion was the first published example of the microbiological hydroxylation of a steroid sulfate (173).

Except for a few modifications the methods used for the isolation and identification of the urinary metabolites were the same as those described by Ruse and Solomon (7), and they proved to be quite effective in these experiments. The identity of the metabolites isolated was established by conventional means employing various physical methods of analyses such as infrared spectroscopy, mp and mmp determinations. In a few instances identification of a metabolite was achieved with the aid of nuclear magnetic resonance spectroscopy and mass spectroscopy (courtesy of Dr. C. Djerassi and Dr. J. Fishman).

Some of the metabolites isolated from pregnancy urine and the urine of the normal male and female were present in such small quantities that a different approach had to be employed for their identification. This method, herein called the Isotope Ratio Procedure, involved acetylation of the metabolite with labelled acetic anhydride and chromatography of the product on several systems until the $^{3}H/^{14}C$ ratio was constant on two successive chromatograms. At this stage the residue was mixed with authentic labelled acetate prepared from the metabolites isolated from the first experiment and this gave a new $^{3}H/^{14}C$ ratio. The labelled acetate, previously shown to be radiochemically pure by chromatography, contained either ^{3}H alone or ^{3}H and ^{14}C, and the mixture was then chromatographed in two or more systems. When the final $^{3}H/^{14}C$ ratio was the same as that predicted after the addition of labelled acetate, radiochemical homogeneity of the metabolite was established. With this type of procedure the specific activity of a number of metabolites was measured and their radiochemical purity was demonstrated.

In several instances a sufficient quantity of a urinary metabolite was isolated to determine its endogenous specific activity by direct crystallization and formation of a derivative. For example, estriol isolated from the glucosiduronidate fraction of pregnancy urine had sufficient weight to permit direct crystallization and derivative formation. Where insufficient weight was obtained for further processing, for example, estriol and Hirschmann's triol isolated from the sulfate fraction of pregnancy urine, the specific activity obtained after the first crystallization was checked by adding carrier steroid to an aliquot of the crystalline metabolite and recrystallization of the mixture and of a derivative prepared

from the final crystallization. In some instances when carrier acetate was available the specific activity of the metabolite isolated in small quantity was determined by the use of the procedure described by Siiteri (165).

The urinary conjugates in the studies described were cleaved first by solvolysis and then by β-glucuronidase hydrolysis. It is therefore possible that the glucosiduronidate fraction had some of the metabolites containing two or more secondary alcohol groups which were excreted in the urine conjugated to both sulfuric and glucosiduronic acids. In one experiment the urinary conjugates were simultaneously hydrolyzed with Glusulase (Experiment 3).

It is interesting to note that in every experiment where the unconjugated steroid was injected the excretion of radioactivity in the 5-day urine collection was more than 60%. Of this amount, over 90% was present in the urine of the first day, indicating that the urinary metabolites resulting from the injected steroid were cleared rapidly. On the other hand, when steroid sulfates were injected the total excretion of radioactivity was not as great and the distribution in the conjugated fractions of urine was also different in that there was a slightly larger portion excreted in the glucosiduronidate fraction. In these instances, the radioactivity was excreted at a slower rate in the urine. In all the studies described the radioactivity excreted as glucosiduronidates was greater than that excreted as sulfates.

The instability of α-ketols under alkaline conditions has been known for a long time. It was therefore imperative that strong alkalis be avoided in the preparation of the neutral extracts of urine in order to prevent degradation of the α-ketols expected as urinary metabolites. Consequently,

a weak alkali, 5% $NaHCO_3$, was used in the preparation of the neutral extracts. In such an extraction scheme the urinary estrogens remained in the organic phase (ethyl acetate), and were processed together with the neutral steroids.

Interest in the metabolism of 16α-hydroxysteroids stemmed from the fact that large amounts of 16α-hydroxydehydroisoandrosterone sulfate are elaborated by the human fetus as is evidenced by the high plasma titers of this steroid taken from the umbilical vein and artery at term (58,59,61). The high levels of 16α-hydroxydehydroisoandrosterone sulfate found in the umbilical vein indicated that not all of the steroid reaching the placenta was hydrolyzed and then aromatized to form estriol. It therefore seemed reasonable that some of the 16α-hydroxydehydroisoandrosterone sulfate which was not aromatized would be transported to the maternal circulation where it would be metabolized and excreted as urinary products. With this as a working hypothesis we began to study the metabolism of 16α-hydroxysteroids. The first task was to determine the nature of the urinary metabolites and to isolate sufficient material for use in further investigations.

Very few 16α-hydroxylated C-19 steroids are available from commercial sources or from investigators who had reported their isolation. The first experiment was therefore designed to obtain sufficient weight in the urinary metabolites for the purpose of identification. After the oral ingestion of 600 mg of 16α-hydroxydehydroisoandrosterone and the simultaneous intravenous injection of the labelled steroid it was possible to isolate and identify a number of metabolites which are listed in Table 81. This table shows the specific activities of the various metabolites and the amounts isolated expressed as a percentage of the injected dose. Three of these are known urinary metabolites, namely, 16α-hydroxydehydroisoandrosterone (19-28),

TABLE 81

Specific Activities of Metabolites Isolated from Normal Male Urine after the Injection of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone and the Oral ingestion of 600 mg of carrier steroid

Metabolite	Sulfate	% of Injected Radioactivity	Glucosiduronidate	% of Injected Radioactivity*
	dpm/mgx10^4		dpm/mgx10^4	
16α-Hydroxydehydroisoandrosterone	1.75	2.8	8.36	0.7
16α-Hydroxyetiocholanolone	2.49	0.1	2.26	1.5
16α-Hydroxyandrosterone	5.06	0.6	4.79	5.1
3α,16α-Dihydroxyandrost-5-en-17-one	5.32	0.7	5.29	12.0
Androst-5-ene-3α,16α,17β-triol	4.99	0.1	5.26	0.4
Androstane triol	5.05	0.2	-	-
Hirschmann's triol	1.80	0.3	-	-
16α-Hydroxyandrostenedione	-	-	4.40	0.1
16α-Acetoxy,3α-hydroxyandrost-5-en-17-one	-		4.96	1.4

*These values were computed from the specific activities and weights of the crystalline metabolites and the total radioactivity recovered in the urine.

androstanetriol (32) and Hirschmann's triol (20,22,27,29-31). The other six metabolites, namely, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 16α-hydroxyandrostenedione, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3α,16α,17β-triol and 16α-acetoxy,3α-hydroxyandrost-5-en-17-one, have not hitherto been described as urinary steroids.

Except for androstanetriol and Hirschmann's triol from the sulfate fraction and 16α-hydroxyandrostenedione and 16α-acetoxy,3α-hydroxyandrost-5-en-17-one from the glucosiduronidate fraction, all the metabolites identified were isolated from both fractions of the urine. 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 most abundant urinary metabolite of 16α-hydroxydehydroisoandrosterone was found to be 3α,16α-dihydroxyandrost-5-en-17-one. The identification of this metabolite was partially accomplished by infrared analysis, chemical tests (B.T. positive, U.V. negative, 77% H_2SO_4 in ethanol positive and Zimmerman negative) and acetylation with ^{14}C-acetic anhydride (two acylable hydroxyl groups). In the N.M.R. spectra (Figure 8, p. 65, courtesy of Dr. J. Fishman) there was in the unacetylated compound, a 3β-proton absorption peak at 4.08 ppm which shifted upfield, 5.03 ppm, in the acetate. When compared to 16α-hydroxydehydroisoandrosterone there was no change in the absorption peaks due to the 6-proton and 16β-proton. Confirmatory evidence for the structure of 3α,16α-dihydroxyandrost-5-en-17-one was obtained by chemical conversion to etiocholanetriol. Since this metabolite could be converted with $NaBH_4$ to a second more polar unknown, the latter was designated as androst-5-ene-3α,16α,17β-triol.

The least polar unknown metabolite isolated in these studies was ultimately identified as 16α-acetoxy,3α-hydroxyandrost-5-en-17-one. The first indication that this unknown was a monoacetate was provided by the mass spectrum (courtesy of Dr. C. Djerassi), which indicated that it had a mass number of 346, consistent with the monoacetate of a steroid such as

16α-hydroxydehydroisoandrosterone (304 + 42). On acetylation of 3α,16α-dihydroxyandrost-5-en-17-one with ^{14}C-acetic anhydride two products were obtained. From the $^{3}H/^{14}C$ ratios of the products it was concluded that one of them was a monoacetate. This monoacetate had the identical infrared spectrum as that of the non-polar metabolite mentioned above. In order to determine the location of the acetate group a number of chemical tests was performed on a solution of the isolated metabolite. These tests indicated that the unknown was B.T. positive only after 5 minutes whereas 16α-hydroxydehydroisoandrosterone gave a positive reaction within seconds. In addition, a slight pink color was observed when the unknown was reacted with 77% H_2SO_4 in ethanol. These tests suggested that the acetate was at C-16. Definitive data concerning the location of the acetate was furnished by the N.M.R. spectrum. This showed a peak at 4.08 ppm corresponding to the absorption of an equatorial 3β-proton in the presence of a 3α-hydroxyl group. An upfield shift to 5.03 ppm due to the presence of a 3α-acetoxy group was not observed. However, an upfield shift of the absorption peak from 4.42ppm to 5.50 ppm due to the 16β-proton was observed, indicating that the 16α-hydroxyl group was substituted. These data together permitted the assignment of the structure of 16α-acetoxy,3α-hydroxyandrost-5-en-17-one to the non-polar metabolite.

Although 11-dehydrocorticosterone acetate has been isolated from human plasma (174), urinary steroid acetates (175) have been considered as artifacts encountered in the course of isolation when the Girard reagent was used (176). Recently, Schubert and Wehrberger (177) reported the isolation of 3β-acetoxyandrost-5-ene-7,17-dione from the unconjugated fraction of a large pool of human urine. In the study described above, 16α-acetoxy, 3α-hydroxyandrost-5-en-17-one was isolated from the glucosiduronidate fraction of urine under conditions where trans-esterification seems unlikely to have occurred. It is possible that this urinary acetate was isolated because

nothing more basic than 5% $NaHCO_3$ was used to prepare the neutral extracts. It would appear that this acetate is excreted as a conjugate with glucosiduronic acid at C-3, a situation analogous to that found in lower mammals (178-181). In these studies, estra-1,3,5(10)-trien-3-ol-17α-yl-2'-acetamido-2'-deoxy-β-D-glucopyranoside was isolated from rabbit's urine (178, 179) and phenylmercapturic acid was isolated from the urine of rabbits after the administration of benzene (180). More work is in progress to determine whether the 16α-acetoxy,3α-hydroxyandrost-5-en-17-one is indeed a normal urinary metabolite.

As can be seen from the percentage conversion in Table 81, the most abundant urinary metabolite isolated had a 3α-hydroxy-5-ene grouping. This indicates that the presence of a 16α-hydroxyl group partially interferes with the Δ^4-isomerase enzyme which would normally play a role in the conversion of a β,γ-unsaturated alcohol to an α,β—unsaturated ketone. That a 3-keto-5-ene steroid may be an intermediate in the formation of a 3α-hydroxy-5-ene steroid was suggested by the studies of Fukushima et al (182) who demonstrated the conversion of androst-5-ene-3,17-dione but not of dehydroisoandrosterone, to urinary 3α-hydroxyandrost-5-en-17-one. Previously, Fukushima et al (37) had isolated a sizable amount of pregn-5-ene-3α,16α,20α-triol from the urine of a patient with adrenal carcinoma. Although large quantities of dehydroisoandrosterone were isolated from the urine of this patient, 3α-hydroxyandrost-5-en-17-one could not be detected. The isolation of 3α,16α-dihydroxyandrost-5-en-17-one in our study is added proof that the 16α-hydroxyl group on a steroid molecule inhibits the Δ^4-isomerase enzyme.

With the exception of androst-5-ene-3α,16α,17β-triol, androstanetriol and 16α-acetoxy,3α-hydroxyandrost-5-en-17-one, all of the metabolites isolated from the urine of the normal male given the steroid per os, were present

in the urine of a pregnant female who was given an intravenous injection of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone contained in 310 μg. In this experiment, estriol was isolated from the sulfate and glucosiduronidate fractions. As shown in Table 82, the specific activities of the urinary 16α-hydroxydehydroisoandrosterone from the sulfate fraction and the urinary

TABLE 82

Metabolites Isolated from the Urine of a Pregnant Subject after the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone

Metabolite	Specific Activity (dpm/μg) Sulfate	Glucosiduronidate	% Conversion of Injected Dose
16α-Hydroxydehydroisoandrosterone	41	390	2.9
16α-Hydroxyandrosterone	250*	1710	5.9
16α-Hydroxyetiocholanolone	-	1190	6.0
16α-Hydroxyandrostenedione	-	1840	1.9
3α,16α-Dihydroxyandrost-5-en-17-one	-	3060	9.5
Hirschmann's triol	68	-	0.9
Estriol	27	44	6.0

*Radiochemical purity of this metabolite was not established because of the small amount of radioactivity isolated.
-These metabolites were not isolated.

estriol from both fractions were similar. The low specific activity of 16α-hydroxydehydroisoandrosterone indicates that a part of the injected steroid was transported to the placenta and possibly to the fetus where it mixed with a large pool of endogenously formed steroid prior to being converted to estriol. Some of this diluted 16α-hydroxydehydroisoandrosterone was then transported to the maternal circulation along with the estriol formed from

it. Large amounts of 16α-hydroxydehydroisoandrosterone do not seem to enter the maternal circulation because if this were so, then the specific activities of 16α-hydroxyetiocholanolone, 16α-hydroxyandrosterone, 16α-hydroxyandrostenedione and 3α,16α-dihydroxyandrost-5-en-17-one would approach that of estriol instead of being considerably higher. On the other hand, the 16α-hydroxydehydroisoandrosterone may be entering the maternal circulation as the sulfate and is excreted as such without being converted to the unconjugated form. The high specific activities of the metabolites in the glucosiduronidate fraction may be a reflection of peripheral metabolism in the maternal compartment.

Although a sizable conversion to estriol was observed in this experiment the major single urinary metabolite was 3α,16α-dihydroxyandrost-5-en-17-one (Table 82). It is of interest to note that when 16α-hydroxydehydroisoandrosterone was injected into the pregnant female the majority of metabolites were isolated from the glucosiduronidate fraction. As was mentioned earlier, this fraction could also contain metabolites conjugated with both sulfuric and glucosiduronic acids. Metabolites such as 16α-hydroxyandrostenedione, however, are excreted in the urine conjugated with glucosiduronic acid, a situation analogous to that found for 16α-hydroxyprogesterone (33).

Ward and Engel (183) have recently demonstrated the conversion of androst-4-ene-3,17-dione to 3β-hydroxyandrost-5-en-17-one by an acetone powder of sheep adrenal microsomes fortified with reduced diphosphopyridine nucleotide. Rosner et al (184) have reported that the analogous reduction of (4-^{14}C)-progesterone to 3β-hydroxypregn-5-en-20-one by homogenates of rabbit testes but the demonstration was not as unambiguous as the results obtained by Ward and Engel (183). These findings demonstrated the possi-

bility of a reversibility of the 3β-hydroxysteroid dehydrogenase-isomerase system but it remains to be determined whether these reactions occur under in vivo conditions. Since the Δ^4-isomerase was partially blocked by the 16α-hydroxyl group it seemed reasonable that with such a steroid the above reaction might be confirmed in vivo. In order to determine whether Δ^4-3-keto steroids could give rise to urinary 3α,16α-dihydroxy-androst-5-en-17-one, a mixture of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone and (4-^{14}C)-16α-hydroxyandrostenedione (^{3}H/^{14}C = 11.4), was injected intravenously into a subject in the third trimester of pregnancy and the urinary metabolites were isolated after Glusulase hydrolysis of the conjugates. As can be seen from the data in Table 83, 3α,16α-dihydroxyandrost-5-en—17-one contained no ^{14}C, indicating that 16α-hydroxyandrostenedione

TABLE 83

Urinary Metabolites Isolated after the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone and (4-^{14}C)-16α-Hydroxyandrostenedione to a Pregnant Female (^{3}H/^{14}C = 11.4)

Metabolite	Specific Activity (dpm ^{3}H/μg)	^{3}H/^{14}C	% Conversion of Injected Dose	
			^{3}H	^{14}C
16α-Hydroxyandrostenedione	520	3.2	2.1	6.4
3α,16α-Dihydroxyandrost-5-en-17-one	2180	∞	13.1	-
16α-Hydroxydehydroisoandrosterone	91	∞	1.3	-
Estriol	31	10.1	7.4	7.9

did not contribute to the formation of the 3α-hydroxy-5-ene steroid.

In the same study the urinary estriol isolated had about the same

$^{3}H/^{14}C$ ratio, 10.1, as that of the injected steroids, 11.4. In addition, the specific activities in the isolated estriol and 16α-hydroxydehydroisoandrosterone were low. It is therefore possible that both of the injected steroids entered the pool of estriol precursors in the feto-placental unit in equal proportions and that all the 16α-hydroxydehydroisoandrosterone was converted into 16α-hydroxyandrostenedione prior to aromatization. Support for this concept comes from the data of Dell'Aqua et al (185) who found that 16α-hydroxydehydroisoandrosterone and 16α-hydroxyandrostenedione were equally well converted to estriol by the perfused human placenta at midterm. However, the slightly lower $^{3}H/^{14}C$ ratio of the isolated estriol may be attributed to further aromatization of 16α-hydroxyandrostenedione in the fetal liver.

The demonstration that 16α-hydroxyandrostenedione can be converted to estriol has been accomplished using in vitro techniques such as incubation of placental microsomes (130) and perfusion of the midterm (185) and term (132,133) placenta. These findings are confirmed and extended by the results of our in vivo study.

Having determined that 16α-hydroxyandrostenedione was efficiently converted to estriol but that it did not serve as a precursor of 3α,16α-dihydroxy-androst-5-en-17-one, experiments were devised to determine whether dehydroisoandrosterone sulfate could be 16α-hydroxylated and serve as a precursor of the urinary metabolites previously isolated. In the next experiment we administered a mixture of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone and (4-^{14}C)-dehydroisoandrosterone sulfate intravenously to a normal subject in the third trimester of pregnancy. From the specific activities and $^{3}H/^{14}C$ ratios of the urinary metabolites shown in Table 84, it can be deduced that dehydroisoandrosterone sulfate is 16α-hydroxylated in the feto-placental unit and is subsequently converted to estriol.

TABLE 84

Specific Activities of Urinary Metabolites Isolated after the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone and (4-^{14}C)-dehydroisoandrosterone sulfate, $^{3}H/^{14}C$ = 5.1, to a Pregnant Subject

Metabolite	Sulfate dpm ^{3}H/μg	$^{3}H/^{14}C$	Glucosiduronidate dpm ^{3}H/μg	$^{3}H/^{14}C$
16α-Hydroxyandrostenedione	-	-	739	191
3α,16α-Dihydroxyandrost-5-en-17-one	-	-	1720	∞
16α-Hydroxydehydroisoandrosterone	41	2.7	1620	∞
Hirschmann's triol	47	3.2	-	-
Estriol	24	3.0	32	7.5

Dehydroisoandrosterone sulfate does not seem to be a precursor of urinary 16α-hydroxyandrostenedione, 3α,16α-dihydroxyandrost-5-en-17-one or 16α-hydroxydehydroisoandrosterone isolated from the glucosiduronidate fraction. Since these three metabolites have much higher specific activities than the others shown in Table 84, it is probable that they were formed by peripheral metabolism in the maternal compartment. We would have to conclude that in this study dehydroisoandrosterone sulfate or dehydroisoandrosterone is not 16α-hydroxylated to any great extent in the maternal compartment since it has been shown that dehydroisoandrosterone sulfate is in metabolic equilibrium with dehydroisoandrosterone (186). This conclusion conflicts with the reported data of Fotherby et al (19) who administered 100 mg of dehydroisoandrosterone intramuscularly to a 45-year old, ovariectomized, adrenalectomized woman and obtained chromatographic evidence for the presence of 16α-hydroxydehydroisoandrosterone in the urine. This metabolite was not present in the urine prior to administration of dehydroisoandrosterone.

On closer examination of the data reported by Fotherby et al it is evident that the X and Y components on paper chromatograms need not be 16α-hydroxylated metabolites of dehydroisoandrosterone.

While the metabolites of this experiment were being processed, Siiteri and MacDonald (5) reported the results of four experiments in which (4-^{14}C)-dehydroisoandrosterone sulfate and (7α-^{3}H)-16α-hydroxydehydroisoandrosterone were injected simultaneously to pregnant women. It was not evident from their data whether the 16α-hydroxydehydroisoandrosterone, Hirschmann's triol and estriol identified, were excreted as sulfates or glucosiduronidates but the ^{3}H/^{14}C ratios obtained in relation to the ratio of the injected steroids were analogous to those found in our study in that the 16α-hydroxydehydroisoandrosterone and Hirschmann's triol had lower ^{3}H/^{14}C ratios than the ratio in the injected steroids, while the estriol had a higher ^{3}H/^{14}C ratio. The authors suggested that the biosynthetic sequence, dehydroisoandrosterone sulfate ⟶ 16α-hydroxydehydroisoandrosterone sulfate ⟶ androst-5-ene-3β,16α,17β-triol sulfate ⟶ estriol, is operative during late pregnancy but their data could not support this sequence. More definite evidence for this conversion sequence was furnished in our study by the similar specific activities of 16α-hydroxydehydroisoandrosterone, androst-5-ene-3β,16α,17β-triol and estriol isolated from the sulfate fraction. That androst-5-ene-3β,16α,17β-triol can be converted to estriol was demonstrated both *in vitro* by placental microsomes (129) and *in vivo* by injecting the labelled steroid into the umbilical vein of the intact feto-placental unit at midpregnancy (187).

Since 16α-hydroxylation of dehydroisoandrosterone sulfate seemed to be an extensive process it was of interest to study the metabolism of

16α-hydroxydehydroisoandrosterone sulfate in late pregnancy. Consequently, a subject in the third trimester of pregnancy was injected intravenously with a mixture of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone sulfate and dehydroisoandrosterone sulfate, $^{3}H/^{14}C$ = 10.4. After the second day of urine collection the subject went into labour so the results obtained in Table 85 may not be a true indication of the events occurring in pregnancy, even though the excretion of radioactivity seems to be the same (Table 49, p.116). This experiment is currently being repeated.

TABLE 85

Urinary Metabolites Isolated after the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone sulfate and (4-^{14}C)-Dehydroisoandrosterone sulfate, $^{3}H/^{14}C$ = 10.4, to a Pregnant Female

Metabolite	Specific Activity (dpm $^{3}H/\mu g$)			
	Sulfate	$^{3}H/^{14}C$	Glucosiduronidate	$^{3}H/^{14}C$
16α-Hydroxyandrostenedione	-	-	223	41.2
3α,16α-Dihydroxyandrost-5-en-17-one	-	-	434	38.4
16α-Hydroxydehydroisoandrosterone	1120	64	-	-
Hirschmann's triol	738	47	-	-
Estriol	51	12.7	52	20.8

It is apparent from the data in Table 85 that dehydroisoandrosterone sulfate can give rise to urinary 16α-hydroxyandrostenedione and 3α,16α-dihydroxyandrost-5-en-17-one. This was not observed in the previous experiment where the $^{3}H/^{14}C$ ratios may have been so high that radioactivity due to ^{14}C was masked. The high specific activity of 16α-hydroxydehydroisoandrosterone in the sulfate fraction maybe due to the renal clearance of a portion of the

injected steroid sulfate prior to mixing and metabolism.

It is interesting to note that the estriol isolated from the previous study as well as this one, had similar specific activities in both the sulfate and glucosiduronidate fractions. However, the $^{3}H/^{14}C$ ratios of the estriol isolated from the glucosiduronidate fraction were greater by a factor of 2 than the ratio of estriol isolated from the sulfate fraction. These high ratios may be due to the presence in this fraction of steroids originally excreted as double conjugates (i.e., 3-sulfate, 16 -glucosiduronidate). In all the pregnant subjects studied the specific activities of estriol were practically the same, indicating that the portion of the injected precursors entering the feto-placental unit is diluted by a pool of intermediates within the unit and then aromatized before being transported to the maternal circulation and excreted in the urine. From the data obtained in Experiment 5 it appears that only estriol was formed in a similar manner as in previous experiments. However, until the extracts obtained from the duplicate study (Table 49, p.116) are processed these data (Table 85) cannot be interpreted fully.

As is evident from the data in Table 86, all the labelled steroids injected in subjects in the third trimester of pregnancy are good precursors of estriol. The average percent conversion of 16α-hydroxydehydroisoandrosterone to urinary estriol was 6.8 while the corresponding value for the conversion of dehydroisoandrosterone sulfate was 6.6 indicating that both these precursors were converted to estriol to approximately the same extent. It should be noted that these values are not corrected for losses which may be from 50% to 80% (5,188). In analogous experiments, Siiteri and MacDonald (5) found that the percent conversion of dehydroisoandrosterone sulfate and 16α-hydroxydehydroisoandrosterone to urinary estriol were 13.9%

TABLE 86

Percent of Injected Radioactivity in the Isolated Metabolites

Experiment	Precursor+	16α-Hydroxy androstenedione	16α-Hydroxydehydroisoandrosterone		3α,16α-Dihydroxyandrost-5-en-17-one		Hirschmann's triol	Estriol	
		G++	S++	G	S	G	S	S	G
1	16 D	0.1	2.8	0.7	0.7	12.0	0.3	-*	-
2	16 D	1.9	0.2	1.3	-	5.1	0.9	0.2	5.9
3***	16 D	2.1	1.3		13.1		-	7.4	
	16 Δ^4	6.4	0.0		0.0		-	7.9	
4	16 D	2.3	2.2	0.1	-	6.0	1.5	0.6	6.2
	DS	0.1	3.7	-	-	0.0	2.5	1.3	4.6
5	16 DS	0.4	3.8	-	-	1.2	6.0	0.5	15.0
	DS	0.2	0.6	-	-	0.3	1.5	0.4	6.8

+ 16 D = (7α-^{3}H)-16α-hydroxydehydroisoandrosterone
16 DS= (7α-^{3}H)-16α-hydroxydehydroisoandrosterone sulfate.
16 Δ^4 = (4-^{14}C)-16α-hydroxyandrostenedione
DS = (4-^{14}C)- dehydroisoandrosterone sulfate

++ S = Sulfate and G = Glucosiduronidate.

* These metabolites were not isolated.

*** The urinary conjugates in this experiment were hydrolysed with Glusulase.

and 20.9% respectively. The most efficient precursor of urinary estriol, based on the data in Table 86, is 16α-hydroxydehydroisoandrosterone sulfate. This fact strengthens the hypothesis put forward by numerous investigators that the huge quantities of estriol formed during late pregnancy are derived from such neutral precursors.

In all the studies performed on subjects in the third trimester of pregnancy the conversion of 16α-hydroxydehydroisoandrosterone to 16α-hydroxyandrostenedione was found to be about 2%. After the injection of dehydroisoandrosterone sulfate or 16α-hydroxydehydroisoandrosterone sulfate only a small amount of 16α-hydroxyandrostenedione could be isolated from the urine ($< 0.5\%$). However, when 16α-hydroxyandrostenedione was administered 6.4% could be found in the glucosiduronidate fraction of urine, indicating that this portion of the injected steroid was conjugated with glucosiduronic acid and excreted without being further metabolized.

A sizable conversion of dehydroisoandrosterone sulfate to 16α-hydroxydehydroisoandrosterone and Hirschmann's triol (sulfate fraction) was observed in Experiment 4 and of 16α-hydroxydehydroisoandrosterone sulfate to Hirschmann's triol in Experiment 5 (Table 86). It should be noted that in all of the studies described Hirschmann's triol was not isolated from the glucosiduronidate fraction, indicating that this steroid is probably excreted exclusively as the sulfate. Whether one or more of the hydroxyl groups is conjugated to sulfuric acid cannot be determined from this type of study. Although 16α-hydroxydehydroisoandrosterone was isolated from both the sulfate and glucosiduronidate fractions, this steroid was present in greater quantities in the sulfate fraction. From the total recovery of 16α-hydroxydehydroisoandrosterone in Experiments 1 to 4 it appears that a major portion of the injected steroid is metabolized and excreted as urinary products.

From Table 86 it can be seen that the most abundant neutral metabolite isolated after the injection of 16α-hydroxydehydroisoandrosterone was 3α,16α-dihydroxyandrost-5-en-17-one. It is reasonable to postulate that this is a unique metabolite of 16α-hydroxydehydroisoandrosterone, and thus the production rate of 16α-hydroxydehydroisoandrosterone may be determined. However, the requirements necessary for this type of determination have not as yet been fulfilled. Baulieu et al (43) have demonstrated that in the non-pregnant state, dehydroisoandrosterone sulfate can be peripherally metabolized to 16α-hydroxydehydroisoandrosterone sulfate. In pregnancy, 16α-hydroxydehydroisoandrosterone and its tracer would have different metabolic fates due to physical compartmentalization in the mother and feto-placental unit. It has been well established that 16α-hydroxydehydroisoandrosterone is produced by the fetus (58,59,61,91). Hence it cannot be assumed that a tracer dose of this steroid injected into the maternal circulation would have the same metabolic fate as the steroid elaborated in the fetus. On the other hand, the 3α,16α-dihydroxyandrost-5-en-17-one may be a unique metabolite of the maternal compartment as exemplified by the high specific activities of this steroid when compared to those of 16α-hydroxydehydroisoandrosterone (Tables 82,83,84). These problems of multiplicity of precursors of urinary metabolites and of physical compartmentalization of the compounds may be overcome using the methods described by Gurpide et al (189). Such studies are already in progress. After the simultaneous injection of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone sulfate and (4-^{14}C)-16α-hydroxydehydroisoandrosterone to normal and pregnant subjects, the rates of interconversion between the two steroids, their rates of metabolism and production rates can be calculated from the cumulative specific activities of the urinary metabolites. Such data will become available.

The first two studies with 16α-hydroxydehydroisoandrosterone described above were carried out on a pregnant subject and a normal male given a large oral dose of carrier steroid. It therefore became necessary to determine whether the metabolism of 16α-hydroxydehydroisoandrosterone would be the same in normal subjects. Thus a tracer dose of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone was given to a normal male and a female in the luteal phase of the menstrual cycle, and the urinary metabolites were isolated and identified. Although we obtained chromatographic evidence for the presence of most of the metabolites isolated in the first two studies, we were unable to establish the identity of some of them because of technical difficulties encountered during the course of isolation. These difficulties mainly involved acetylation of metabolites with labelled acetic anhydride. In Table 87 are shown the metabolites which have been identified in these two studies. The percent conversions of the injected dose

TABLE 87

Urinary Metabolites Isolated after the Injection of (7α-^{3}H)-16α-Hydroxydehydroisoandrosterone to Normal Subjects

	Specific Activity (dpm/μg)			
	Sulfate		Glucosiduronidate	
Metabolite	Male	Female	Male	Female
16α-Hydroxydehydroisoandrosterone	234	169	-	5800
3α,16α-Dihydroxyandrost--5-en-17-one	-	294*	8520	5760
16α-Hydroxyandrosterone	1110	663	8570	5560
16α-Hydroxyetiocholanolone	-	558	3150	1560

*Radiochemical purity of this metabolite was not established because of the small amount of radioactivity remaining.

were not determined because the losses incurred during isolation were much

greater than in previous studies. As can be seen from the data in Table 87, the specific activities of the metabolites in the sulfate fraction were much lower than those in the glucosiduronidate fraction. The rather low specific activities of 16α-hydroxydehydroisoandrosterone in both studies indicate that this steroid is probably excreted as the sulfate from the adrenal or dehydroisoandrosterone may be 16α-hydroxylated as the sulfate. Although 16α-hydroxydehydroisoandrosterone has been isolated from normal male urine (19-22), it was not evident from the published data that this steroid was excreted as a sulfate or glucosiduronidate. In our studies we isolated weighable amounts of 16α-hydroxydehydroisoandrosterone from the sulfate fraction but not from the glucosiduronidate fraction. Thus it seems that 16α-hydroxydehydroisoandrosterone sulfate is a normal urinary metabolite.

Estriol found in the urine of normal women (17,18) is probably derived from the metabolism of estrone and estradiol. We had obtained chromatographic evidence for the presence of labelled estriol in the glucosiduronidate fraction of normal female urine (Experiment 7). However, it was not possible to establish radiochemical purity of this metabolite after acetylation with ^{14}C-acetic anhydride and crystallization with carrier acetate (Table 80).

From the specific activities of urinary 16α-hydroxydehydroisoandrosterone obtained after the injection of labelled 16α-hydroxydehydroisoandrosterone to a normal, non-pregnant female (169 dpm/μg) and to a pregnant female (41 dpm/μg) it seems reasonable to speculate that at least four times as much 16α-hydroxydehydroisoandrosterone is being transported from the feto-placental unit to the maternal compartment as is being elaborated by the normal adrenal gland. Because of physical compartmentalization it is not valid to predict the events occurring in the fetus by extrapolation of the results

obtained after administering labelled steroid into the mother. However, differences in metabolism of a particular steroid between the pregnant and non-pregnant states can be attributed to the presence or absence of the feto-placental unit.

A more direct method of determining the metabolic fate of a steroid in late pregnancy would be to inject the same steroid with two labels, one into the umbilical vein and the other into the antecubital vein, and to isolate the urinary metabolites. Such an experimental design was possible when a patient was admitted for therapeutic abortion at midpregnancy (190). However, similar procedures cannot for obvious reasons be performed during late pregnancy. Until these problems are solved, *in vivo* steroid metabolism in the fetus approaching term can only be studied by administering labelled steroid into the maternal compartment.

Various types of experimental design have been employed in studying steroid metabolism in the isolated fetus, placenta and feto-placental unit at midterm. In our studies we have tried to gain some more knowledge regarding metabolism in the third trimester of pregnancy, but our experimental design does not permit a true evaluation of the differences in metabolism between the three compartments present in pregnancy. From the data obtained by the elegant work of several investigators we know that extensive metabolism of steroids occurs in the fetus. However, the physiological significance of the complex interplay of steroid metabolism among mother, placenta and fetus remains a difficult problem to be solved.

SUMMARY AND CONCLUSIONS

Labelled 16α-hydroxylated C-19 steroids were prepared by the microbiological 16α-hydroxylation of the appropriate substrate with Streptomyces roseochromogenus. The preparation of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone, (7α-^{3}H)-16α-hydroxydehydroisoandrosterone-3-sulfate and (4-^{14}C)-16α-hydroxyandrostenedione was described.

After the intravenous injection of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone and the ingestion of 600 mg of carrier steroid the urinary conjugates were hydrolysed by solvolysis and then by β-glucuronidase. From the sulfate fraction the following metabolites were isolated and identified: 16α-hydroxydehydroisoandrosterone, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 3α,16α-dihydroxyandrost-5-en-17-one, androstanetriol, androst-5-ene-3β,16α,17β-triol and androst-5-ene-3α,16α,17β-triol. From the glucosiduronidate fraction were isolated: 16α-acetoxy,3α-hydroxyandrost-5-en-17-one, 16α-hydroxyandrostenedione, 16α-hydroxyandrosterone, 16α-hydroxydehydroisoandrosterone, 3α,16α-dihydroxyandrost-5-en-17-one, 16α-hydroxyetiocholanolone and androst-5-ene-3α,16α,17β-triol. Only three of these steroids, namely, 16α-hydroxydehydroisoandrosterone, androstanetriol and androst-5-ene-3β,16α,17β-triol, have previously been isolated from human urine.

With the exception of androstanetriol, 16α-acetoxy,3α-hydroxyandrost-5-en-17-one and androst-5-ene-3α,16α,17β-triol, all of the above-mentioned metabolites could be isolated from the urine of a pregnant subject given an intravenous injection of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone. In addition, labelled estriol was isolated from both fractions of the urine.

Urinary estriol produced during late pregnancy can be derived from maternally administered dehydroisoandrosterone sulfate, 16α-hydroxydehydro-

isoandrosterone, 16α-hydroxydehydroisoandrosterone sulfate and 16α-hydroxyandrostenedione. Of these precursors, 16α-hydroxydehydroisoandrosterone sulfate was the most efficient one for the formation of estriol.

The novel 3α-hydroxy-5-ene steroid, 3α,16α-dihydroxyandrost-5-en-17-one, was not derived from 16α-hydroxyandrostenedione and only to a small extent from dehydroisoandrosterone sulfate and 16α-hydroxydehydroisoandrosterone sulfate.

When a mixture of (7α-^{3}H)-16α-hydroxydehydroisoandrosterone and (4-^{14}C)-dehydroisoandrosterone sulfate was injected into a normal subject in the third trimester of pregnancy, urinary 16α-hydroxydehydroisoandrosterone, Hirschmann's triol and estriol from the sulfate fraction all had similar ^{3}H/^{14}C ratios which were lower than the ratio of the injected steroids. On the other hand, the estriol isolated from the glucosiduronidate fraction had a higher ^{3}H/^{14}C ratio than that injected and the other metabolites in this fraction had little or no ^{14}C.

Weighable amounts of 16α-hydroxydehydroisoandrosterone were isolated from the sulfate fraction of the urine of a normal male and a normal non-pregnant female. In these subjects, labelled 16α-hydroxydehydroisoandrosterone was metabolized to urinary 16α-hydroxydehydroisoandrosterone, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone and 3α,16α-dihydroxyandrost-5-en-17-one. Estriol was not identified in the urine of the normal non-pregnant female.

CLAIMS TO ORIGINAL RESEARCH

1. A novel method was developed for the 16α-hydroxylation of dehydroisoandrosterone sulfate (173).

2. A novel method, herein called the Isotope Ratio Procedure, was devised for the identification of minute quantities of urinary metabolites.

3. The following urinary metabolites of 16α-hydroxydehydroisoandrosterone were isolated and identified: 16α-hydroxydehydroisoandrosterone, 16α-hydroxyandrostenedione, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3β,16α,17β-triol, androstanetriol, androst-5-ene-3α,16α,17β-triol and 16α-acetoxy,3α-hydroxyandrost-5-en-17-one. With the exception of 16α-hydroxydehydroisoandrosterone, androstanetriol and androst-5-ene-3β,16α,17β-triol, all the other metabolites, a total of six, have not hitherto been isolated from human urine (191).

4. When labelled 16α-hydroxydehydroisoandrosterone was administered to a subject in the third trimester of pregnancy, four of these novel urinary metabolites, namely, 16α-hydroxyandrostenedione, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone and 3α,16α-dihydroxyandrost-5-en-17-one were also isolated (191).

5. It was demonstrated that 16α-hydroxyandrostenedione does not contribute to the formation of urinary 3α,16α-dihydroxyandrost-5-en-17-one, whereas dehydroisoandrosterone sulfate and 16α-hydroxydehydroisoandrosterone sulfate are converted to this metabolite only to a small extent.

6. It was demonstrated *in vivo* that the estriol produced during late pregnancy can be derived from 16α-hydroxyandrostenedione and 16α-hydroxydehydroisoandrosterone sulfate, the latter being the more efficient precursor.

7. The relative contribution of maternally administered 16α-hydroxy-

dehydroisoandrosterone and 16α-hydroxyandrostenedione to the formation of urinary estriol in late pregnancy was found to be the same.

8. It was demonstrated that 16α-hydroxydehydroisoandrosterone in the form of the sulfate was excreted in relatively large amounts in normal male and female urine. In these subjects, 16α-hydroxydehydroisoandrosterone can be further metabolized to identifiable products.

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