THE METABOLISM OF 16α-HYDROXYLATED STEROIDS

by

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ABBREVIATIONS

g	-	gram
mg	-	milligram
ml	-	millilitre
m.p.	-	melting point
m.m.p.	-	mixed melting point
s.a.	-	specific activity
ACTH	-	adrenocorticotrophic hormone
DPN	-	diphosphopyridine nucleotide
TPN	-	triphosphopyridine nucleotide
uC	-	microcurie
mC	-	millicurie

TRIVIAL NAMES

progesterone	- Δ^4-pregnen-3,20-dione
16α-hydroxy-progesterone	- 16α-hydroxy-Δ^4-pregnen-3,20-dione
pregnenolone	- 3β-hydroxy-Δ^5-pregnen-20-one
16α-hydroxy-pregnenolone	- 16α,3β-dihydroxy-Δ^5-pregnene-20-one
isopregnanolone	- 3β-hydroxy-5β,17α-pregnan-20-one
testosterone	- 17β-hydroxy-Δ^4-androstene-3-one
16α-hydroxy-testosterone	- 16α,17β-dihydroxy-Δ^4-androstene-3-one
oestradiol	- $\Delta^{1,3,5(10)}$oestratriene-3,17β-diol
oestriol	- $\Delta^{1,3,5(10)}$oestratriene-3,16α,17β-triol

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INTRODUCTION

The occurrence of neutral 16-hydroxylated steroids in biological materials has been recognized for many years. In 1940, Marker and Turner (1) demonstrated the presence of a 16-hydroxy group in a steroid isolated 7 years previously by Smith et al from pregnant mares' urine (2). It was not until 1945 however that Hirschmann (3) showed the compound to be 5α-pregnan-3β,16α,20β-triol.

The first report of a neutral 16α-hydroxylated steroid in human urine was published by Hirschmann in 1943 (4). He isolated the compound from the urine of a patient with an adrenocortical carcinoma and showed it to be Δ^5-androstene 3β,16ξ,17ξtriol. The orientation of the hydroxyl groups at C16 and C17 was not established until the work of Huffman and Lot (5) and of Goldberg (6) made possible the identification of the steroid as Δ^5-androstene-3β,16α,17β-triol.

In 1945 Hirschmann et al reported the isolation of a trihydroxy steroid from the urine of a patient with the adrenogenital syndrome (7). Later (8) they were able to identify the compound as Δ^5-pregnene-3β,16α,20α-triol. Several years later the same steroid was isolated by

Fotherby from the urine of normal males (9).

In 1953 Lieberman et al reported the isolation of three new 16α-hydroxy-steroids, the isomeric 5α- and 5β-androstane-3α,16α,17β-triols, and 5α-pregnan 3β,16α,20α-triol. The compounds were found in large pools of urine collected from hospital patients with unspecified illnesses (10).

In 1956 Fotherby (11) identified 3β,16α-dihydroxy-Δ^5-androstene-17-one in the urine of normal males. It was extracted after boiling the urine, and was actually isolated as a 3,5-cyclo-6β-hydroxy compound. He demonstrated that the structure arose from the 3-sulphate of the steroid during the boiling. Later (12) the author reported the isolation from the same source of Δ^5-androstene-3β,16β,17β-triol, the only reported example of a neutral 16β-hydroxy compound in human urine. This compound was also isolated in the 3,5-cyclo-6β-hydroxy form, suggesting that it was present in the urine as the sulphate. In 1959, Okada et al (13) reported the isolation of two unusual steroids, 3β,7α,16α-trihydroxy-Δ^5-androstene-17-one and 3β,16α-dihydroxy-Δ^5-androstene-7,17-dione. They were found in the urine of a patient with adrenocortical carcinoma.

More recently Neher et al have reported (14) that the urine of a patient with congenital adrenal hyperplasia contained 3α,16α-dihydroxy-5α-pregnan-20-one and 3α,16α-dihydroxy-5β-pregnan-20-one. The second of these compounds

was found to promote salt excretion by adrenalectomized male rats, and the authors suggested it might be responsible for the patients' salt losing tendency.

In 1961, Hirschmann et al (15) published a report of the isolation of 5α-pregnan-3α,16α,20α-triol and 5β-pregnan-3α,16α,20α-triol from human pregnancy urine. The compounds were found in the neutral extract after β-glucuronidase hydrolysis. The authors pointed out that the weights of the isolated compounds, 10.8 and 1.2 mg respectively, were not in the proportions usually found in the metabolites of C_{21} steroids. They suggest that 16α-hydroxy-progesterone may be the precursor of these compounds, as well as the dihydroxyketones reported by Neher et al. On the same date, Fukushima et al (16) reported the isolation of Δ^5-pregnen-3α,16α,20α-triol from the urine of a patient with widespread adrenocortical carcinoma, the only example reported of a 3α-hydroxy-Δ^5-steroid. The authors raised the possibility that 3β,16α-dihydroxy-Δ^5-pregnene-20-one is a major secretory product in patients with adrenocortical hyperplasia, and that, in keeping with the findings in pregnancy, a portion is metabolized to 16α-hydroxy progesterone. The 3α-hydroxy-Δ^5-steroid, they suggest, could result from overproduction of the intermediate 16α-hydroxy-Δ^5-pregnen-3,20-dione, together with an inhibiting effect of the 16α-hydroxy group on Δ^5-3-keto-steroid

isomerase. There is a report of such an inhibitory effect on the metabolism of certain synthetic corticoids (17). Alternatively they suggest that the unusual metabolite may be derived from an intermediate such as might be found in the metabolism of 3β-hydroxy-Δ^5-androstene-17-one to its 3α-hydroxy metabolites androsterone and etiocholanolone.

It was, therefore, of considerable interest that in 1962 Bongiovanni reported the isolation of 3β,16α-dihydroxy-Δ^5-pregnen-20-one from the urine of infants with deficiency of 3β-ol-dehydrogenase (18). In this condition, as in many cases of adrenocortical carcinoma, the urine is rich in 3β-hydroxy-Δ^5-steroids. The finding lends weight to the suggestions of Fukushima. The author reports excretion of 3-4 mg per day in two cases. The same urines yielded 3β,16α-dihydroxy-Δ^5-androstene-17-one. The compounds were found in neutral extracts after solvolysis of steroid sulphates. Since this report, Reynolds (19) has reported the former compound in the urine of the normal newborn, as well as a neonate with the adrenogenital syndrome. The patients excreted 0.2-0.8 and 4-8 mg per day respectively. In these cases also, the compound was found in the neutral extracts after solvolysis.

Table I is a list of the neutral 16α-hydroxy-steroids isolated from human urine. It is noteworthy that none of the C_{21} steroids have more than 3 oxygen functions.

TABLE I

Neutral 16α-Hydroxy Steroids Isolated from Human Urine

$C_{19}O_3$	3β,16α-dihydroxy-Δ^5-androstene-17-one.
	Δ^5-androstene-3β,16α,17β-triol.
	Δ^5-androstene-3β,16β,17β-triol.
	5β-androstane-3α,16α,17β-triol.
	5α-androstane-3α,16α,17β-triol.
$C_{19}O_4$	3β,7α,16α-trihydroxy-Δ^5-androstene-17-one.
	3β,16α-dihydroxy-Δ^5-androstene-7,17-dione.
$C_{21}O_3$	3β,16α-dihydroxy-Δ^5-pregnen-20-one.
	Δ^5-pregnen-3β,16α,20α-triol.
	Δ^5-pregnen-3α,16α,20α-triol.
	5α-pregnan-3β,16α,20α-triol.
	5α-pregnan-3α,16α,20α-triol.
	5β-pregnan-3α,16α,20α-triol.
	3α,16α-dihydroxy-5α-pregnan-20-one.
	3α,16α-dihydroxy-5β-pregnan-20-one.

Fukushima (16) has suggested that the 16α-hydroxy group may interfere with subsequent hydroxylation in steroid producing tissues. However, it is not known whether the C_{19} steroids were hydroxylated at C16 before or after the loss of the side chain, so that 17α-hydroxylation of 16α-hydroxy steroids remains a possibility. The origin of these steroids is largely unknown. In 1956, Fotherby reported the isolation of 3β,16α-dihydroxy-Δ^5-androstene-17-one from the urine of an oophorectomized, adrenalectomized female after

intramuscular administration of 3β-hydroxy-Δ^5-androstene-17-one (20). The 16α-hydroxy-steroid was not detectable prior to the injection. This is apparently the only demonstration of a precursor of a urinary neutral 16α-hydroxy metabolite in the human subject.

Neutral 16α-hydroxy-steroids have been isolated from a variety of human tissues. In 1961, Neher and Stark (21) reported the isolation of 16α-hydroxy-testosterone from extracts of placenta. The following year Zander et al (22) reported the presence of 16α-hydroxy-progesterone in corpora lutea, and in blood taken from umbilical vessels at the time of delivery. More recently Magendantz and Ryan (23), and Colas et al (24) have described the isolation of 3β,16α-dihydroxy-Δ^5-androstene-17-one from umbilical artery and vein blood. The steroid was present in blood as the 3-sulphate. It has been shown to be a precursor of oestriol in <u>in vitro</u> studies with placental tissues (23). A number of tissues, both foetal and adult, are known to be capable of hydroxylation in the 16α position. In 1958, Engel et al (25) demonstrated the <u>in vitro</u> conversion of oestradiol to oestriol by human foetal liver, to the extent of 3.5 percent. In 1961, Villee et al (26) showed that the foetal adrenal was capable of transforming progesterone to 16α-hydroxy-progesterone, and that this ability was present very early in foetal life. In the same year, Warren and Salhanick

demonstrated the presence of a 16α-hydroxylase in the adult ovary (27) and suggested that the activity was greatest in tissue rich in follicular and thecal elements. The authors suggested, with some reservation, that polycystic ovaries were more active in 16α-hydroxylation than normal ovaries. In 1962, Villee et al (28) reported the conversion of progesterone to 16α-hydroxy-progesterone by homogenates of hyperplastic adrenocortical tissue. In the presence of ACTH, TPN and glucose-6-phosphate, the extent of conversion was a remarkable 21 percent. The following year Acevado et al (29) reported the conversion of progesterone to 16α-hydroxy-Δ^4-androstene-3,17-dione, and of pregnenolone to 3β,16α,17β-trihydroxy-Δ^5-androstene by a mince of foetal testis. Shortly afterwards, Griffiths et al (30) incubated the testes from a patient with the syndrome of testicular feminization and demonstrated 16α-hydroxylation of progesterone. A Sertoli cell tumor found in one testis also possessed 16α-hydroxylase activity. About the same time, Ward and Grant (31) reported 16α-hydroxylation of progesterone by 2 hyperplastic adrenal glands, and an adrenocortical tumor.

In 1963 Longchampt and Alexrod reported the conversion of testosterone to 16α-hydroxy-testosterone by homogenates of foetal adrenal (32). More recently, Villee has reported the results of incubations of hyperplastic adrenocortical tissue with progesterone and pregnenolone (33). In the

progesterone experiments the author found 16α-hydroxy-progesterone and 16α,17α-dihydroxy-progesterone, although the evidence for the latter compound is incomplete. In the pregnenolone studies, 16α-hydroxy-pregnenolone was identified, except in those homogenates to which DPN was added. In the latter case 16α-hydroxy-progesterone was found.

Since the isolation of 3β,16α,20β-trihydroxy-5α-pregnan-20-one from pregnant mares' urine (2), few neutral 16α-hydroxy steroids of animal origin have been reported. In 1948, Schneider and Mason (34) demonstrated the in vitro conversion of dehydroepiandrosterone to Δ^5-androstene-3β, 16α,17β-triol by rabbit liver. In 1956 Brooks and Klyne reported the isolation of 5α-androstane-3α,16α-diol and 5α-androstane-3α,16β-diol from mares' urine (35). Soon afterwards, Balker et al (36) isolated radioactive 5α-pregnan-3β,16α,20β-triol from the urine of a pregnant mare given 1-^{14}C-acetate. At about the same time, Rao and Heard (37) showed that hog adrenals possessed the ability to hydroxylate progesterone in the 16α position. In 1958, Neher et al (38) isolated 3β,16α-dihydroxy-5α-pregnan-20-one from hog adrenals. The following year Wettstein et al (39) isolated 16α-hydroxy-progesterone from hog adrenals, in the amount of 150 mg per ton of adrenal tissue. In the same paper the authors reported the conversion by rat liver of progesterone and 16α-hydroxy-progesterone to 3β,16α-dihydroxy-

5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one and 3α,16α-dihydroxy-5α-pregnan-20-one. They also found that bovine adrenals converted progesterone to 16α-hydroxy-progesterone in low yield.

There is a single report in the literature of the metabolism of 16α-hydroxy-progesterone by the human subject. In 1962, Calvin and Lieberman (40) found labelled 3α-hydroxy-17α-pregnan-20-one (isopregnanolone) and 3α-hydroxy-Δ^{16}-5β pregnen-20-one in the urine of a normal male after the administration of ^{3}H-16α-hydroxy-progesterone. They were able to measure the specific activity of urinary isopregnanolone, and to show that its specific activity was reduced by the oral administration of $\Delta^{4,16}$-pregnadiene-20-one. They suggested that 16α-hydroxy-progesterone is metabolized to isopregnanolone by way of a 16-dehydro compound.

MATERIALS AND METHODS

PREPARATION OF SOLVENTS

Methylene dichloride, ethyl acetate, methanol, dioxane and toluene (reagent grade, Fisher Scientific), cyclohexane (practical, Distillation Products Ind.) and acetone (analytical grade, Mallinckrodt) were distilled prior to use. Diethyl ether and benzene (analytical grade, Mallinckrodt) were distilled over KOH. Chloroform (analytical grade, Mallinckrodt) was distilled over K_2CO_3. Tetrahydrofuran (reagent grade, Fisher Scientific) was refluxed over KOH for 2 hours then distilled over KOH. It was used within 24 hours of preparation. Skellysolve B and Skellysolve C (Skelly Oil Co.) were used as received. Absolute ethanol (Gooderham and Worts) was used as received. All solvents were stored in brown bottles. Pyridine (Merck) was distilled over barium oxide in a closed system. Acetic anhydride was distilled over fused sodium acetate in a closed system. Pyridine and acetic anhydride were kept in glass-stoppered bottles and stored in desiccators.

MEASUREMENT OF RADIOACTIVITY

Aliquots of samples to be counted were evaporated under nitrogen in 5 dram vials (Wheaton Glass Co.) and dissolved in 5 ml of toluene containing 0.3% (w/v) of 2,5-diphenyl-oxazole (PPO) and 0.01% (w/v) of 1,4-bis-2-(5-phenoxazolyl)-benzene (POPOP). Compounds insoluble in toluene were dissolved in 0.1 or 0.2 ml of methanol prior to addition of toluene phosphor solution. Radioactivity in aqueous solutions was measured by dissolving 1 ml of the solution in 15 ml of dioxane containing 0.7% (w/v) of PPO, 0.06% (w/v) of POPOP and 10% (w/v) of napthalene. Samples were counted for a time sufficient to give a standard deviation of 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.

The model 314X was used at a photomultiplier setting of 1050 volts (HV tap 6) for tritium, and 745 volts (HV tap 3) for carbon-14. The discriminator settings were 10 and 100 in each case. Efficiency of counting was approximately 18% for tritium and 60% for carbon-14. Simultaneous counting of tritium and carbon-14 was done at a voltage of 1000 (HV tap 5). The upper and lower discriminators were set at 10 and infinity. The setting of the middle discriminator was changed from time to time to maintain a suitable ratio

of counts in the two channels. The total tritium and carbon-14 counts were calculated by the discriminator ratio method of Okita et al (41), as modified by Ulick (42). The following equations were used.

$$^{3}H = N_1 - N_2/b$$

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

where N_1 = total counts in the first channel

N_2 = total counts in the second channel

$$a = \frac{^{3}H \text{ in the second channel}}{^{3}H \text{ in the first channel}}$$

$$b = \frac{^{14}C \text{ in the second channel}}{^{14}C \text{ in the first channel}}$$

The middle discriminator was set so that a and b were as close as possible to 0.02 and 2.0 respectively. The actual a and b ratios were determined with every set of vials by counting tritium and carbon-14 standards.

Tritium was counted in the model 3002 at an amplifier gain of 60% and discriminator settings of 50 and infinity. At these settings tritium was counted with an efficiency of 40%. Carbon-14 was counted at the same settings, with an efficiency of 92%. In simultaneous counting, tritium was measured in one channel at 30% gain and discriminator settings of 50 and 330, and carbon-14 in the second channel at 40% gain and discriminator settings of 600 and infinity.

Efficiency of counting was 37% for tritium and 75% for carbon-14. Calculation of tritium and carbon-14 counts was done as above. The a and b ratios in unquenched samples were approximately 0.01 and 4.8 respectively.

Counts of single-labelled compounds were corrected for quenching by the use of internal standards. Vials were recounted after addition of a known amount of radioactivity in 0.1 or 0.2 ml of toluene. Comparison of the increment in counts with the actual number of counts added provided a ratio which was used to correct the original sample count. Double-labelled samples were recounted after the addition of tritium standard, and again after the addition of carbon-14 standard. The values of a and b were calculated from the increments in the two channels following addition of the two standards. The count of a given isotope in the original and supplemented vials was calculated, and then corrected according to the ratio of increment to counts added.

MELTING POINT DETERMINATIONS

All melting points were determined with a Kopfer Block (H.O.Post.Scientific Co.) and were corrected by reference to a curve based on the observed melting points of a series of standards (Fisher Scientific Co.).

INFRARED SPECTROSCOPY

Absorption of radiation in the infrared region was measured with a Perkin-Elmer spectrophotometer. Samples were examined as 1% solutions in CS_2 whenever possible. Samples not soluble in CS_2 were examined as dispersions in KBr discs, using 200-500 ug of steroid and 20 mg of KBr.

PAPER CHROMATOGRAPHY

Strips of Whatman #1 or #3MM filter paper 15 x 42 cms were used. The solvent systems employed were n-heptane: propylene glycol, Skellysolve C:propylene glycol and toluene:propylene glycol, all run as described by Zaffroni (43) and Savard (44). Just prior to application of the sample, the papers were impregnated with propylene glycol: methanol::1:1, and blotted.

Ultra violet absorbing steroids were located by examination in U.V. light, using a viewing box. Other steroids were visualized by spraying the chromatogram with a 10% solution of phosphomolybdic acid in ethanol. The papers were then heated to about 90°C for 1-2 mins. Reacting steroids appeared as blue spots on a green background (45). Radioactive steroids were located with a windowless strip-scanner (Vanguard model 880). The gas was 99.05% helium and 0.95% isobutane (Matheson).

Areas of paper containing steroid to be eluted were cut in small squares, covered with methanol and allowed to stand for 18-24 hrs. After removal of the methanol, the paper was re-extracted twice with methanol for 5 to 10 mins. The methanol was filtered, pooled and evaporated *in vacuo* at 40°C. The residue was then dissolved in ethyl acetate and washed twice with 1/5 volume of water to remove any traces of stationary phase. The ethyl acetate was dried over Na_2SO_4 and evaporated *in vacuo* at 40°C.

DETERMINATION OF PARTITION COEFFICIENTS

The partition coefficient of a steroid in a given system was determined by dissolving a known weight of the steroid in equal volumes of equilibrated upper and lower phases. The upper phase was evaporated and the residue weighed. Coefficients were calculated using the expression

$$K = \frac{\text{weight in upper phase}}{\text{total weight - weight in upper phase}}$$

CELITE COLUMN CHROMATOGRAPHY

Celite (Johns-Manville, #545) was washed with 50% HCI for 24 hrs., with tap water for 72 hours, with distilled water, and finally with methanol. It was then dried in air at room temperature for 48 hours, or in an oven at 80-90°C for 18 hours, and stored in glass containers. The solvent

systems used for chromatography were:

A. n-hexane:methanol:water::100:90:10

B. n-heptane:ethyl acetate:methanol:water::50:50:65:35

C. n-hexane:ethyl acetate:methanol:water::50:50:65:35

D. benzene:cyclohexane:methanol:water::1:2:3:3 (46).

Celite was used in the ratio of 0.5 to 1.0 g per mg of extract. All columns 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.

When possible, samples were dissolved in mobile phase for application to the column. Those samples not soluble in mobile phase were dissolved in stationary phase and mixed with celite, which was then packed on the top of the column. In a few instances, the extracts were dissolved in small volumes of stationary phase and applied directly to the column. Mobile phase was delivered from a glass reservoir attached to the top of the column by standard taper joints. The effluent was collected in tubes in an automatic fraction collector (Buchler Instruments, or LKB).

The hold-back volumes of the columns were determined by measuring the volume of solvent required to elute the dye

Sudan IV (47). As our experience with the packing of columns increased, the hold-back volumes became quite constant at about 1.2 ml per g of celite. All celite columns were run at room temperature.

SILICA GEL COLUMN CHROMATOGRAPHY

Silica gel, 100-200 mesh, was obtained from Davison Chemical Co. and used as received. A slurry of silica gel in the starting solvent was transferred to a glass column, which was tapped vigorously to ensure even settling of the silica gel and removal of air. Samples were applied as solutions in the starting solvent. The effluent from the smaller columns was collected in fractions of 1 ml per g of adsorbent. Fractions from the large columns were proportionately much smaller.

ALUMINA COLUMN CHROMATOGRAPHY

Harshaw alumina (200 mesh) was neutralized according to the procedure described by Solomon et al (48). After drying, the alumina was deactivated by the addition of 5 ml of water per 100 g. The alumina was poured slowly into a glass column containing the starting solvent. The column was tapped vigorously during packing. Fractions of 1 ml per g of alumina 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 1 mm. using a Research Specialties Co. spreader. The plates were dried in air for one hour or more, and then heated to 120°C for one-half hour. Samples were spotted on a 10 cm line 2 cms 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 UV light or by spraying with phosphomolybdic acid in ethanol. After spraying with phosphomolybdic acid the plates were warmed to about 35°C for 20 to 30 mins. Steroids appeared as blue spots on a yellow background. When necessary, radioactivity was located by measuring the radioactivity in the eluates of 2 cm sections of the silica gel.

The silica gel to be eluted was transferred to a sintered glass funnel of 100 ml capacity, covered with ethyl acetate, stirred and allowed to stand for 5 to 10 mins. The ethyl acetate was then filtered off with the aid of vacuum. Extraction was repeated twice, and the pooled ethyl acetate evaporated *in vacuo* at 40°C.

PREPARATION OF STEROIDS FOR INJECTION

Labelled steroids were stored in solution in benzene: methanol (4:1). Suitable aliquots were transferred to sterile vials, using sterile pipettes. Solvents were evaporated under nitrogen and the residues dissolved in 1.0 ml of absolute ethanol. Just prior to injection, 10 ml of sterile isotonic saline was added to the vials. The solutions were injected slowly into the antecubital vein from 20 ml syringes. The syringes were washed once with the subject's blood, which was re-injected. Afterwards the syringe, needle and vial were washed with saline solution and with ethyl acetate, and the radioactivity measured. In the experiments reported here, only insignificant amounts of radioactivity were recovered from the syringes and vials.

HYDROLYSIS OF URINARY STEROID CONJUGATES

Urines not processed immediately after collection were stored in the frozen state. Simultaneous enzymatic hydrolysis of sulphates and glucosiduronidates was accomplished with the use of Glusulase (Endo Labs, New Jersey), a mixture containing 100,000 units of β-glucuronidase and 50,000 units of sulphatase per ml. Urine was adjusted to pH 5.2 with 50% sulphuric acid, buffered with 2 volumes percent sodium acetate buffer and treated with the enzyme

mixture. Volumes of Glusulase used are noted in the descriptions of individual experiments.

In one experiment, the steroid residues of the sulphuric acid and glucuronic acid conjugates were extracted separately. Sulphates were cleaved by the solvolytic method described by Burstein and Lieberman (49). Subsequently the glucosiduronidates were hydrolyzed with β-glucuronidase. The details are given in the description of the experiment.

PREPARATION OF NEUTRAL EXTRACTS OF URINE

All neutral extracts of urine were obtained by extraction with ethyl acetate. A volume of ethyl acetate 1.5 to 2.0 times that of the urine was divided among three separatory funnels in the ratio 2:1:1. The urine was passed through the funnels in sequence, using about 1/5 of the urine at a time. The ethyl acetate was then pooled, washed with 0.5N NaOH until pigment removal declined, and with water until the water washes were neutral. The ethyl acetate was then dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* at 40°C. The residue constituted the neutral extract.

DETERMINATION OF SPECIFIC ACTIVITY

Crystalline compounds were weighed accurately in platinum boats, using a micro-balance (Meitler, model

1-912-3X3). Sample weights varied from 0.600 mg to 2.000 mg. The crystals were then transferred quantitatively to counting vials, or to volumetric flasks. The contents of the vials or the residues of aliquots from the volumetric flasks were dissolved in toluene phosphor and counted. Mother liquors from crystallizations were transferred to small (2.5-3.0 g) accurately weighed flasks. After drying and re-weighing, they were transferred to volumetric flasks and aliquots taken for counting.

The balance used was accurate to $\pm$ 10 ug in the weight range of the boats and flasks. In the case of certain urinary metabolites, as little as 180 ug were available for weighing. At this level, errors in weighing were about 5%. Sufficient counts were accumulated to give a standard deviation of 2%. Thus under unfavorable circumstances, weighing and counting involved an error of $\pm$ 10% or more.

FORMATION OF 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^{o}C.

Reactions were stopped in two ways. In the first, the tube was placed in a water bath at 40°C and the solvents evaporated under a stream of nitrogen. Benzene:methanol (1:1) was added and evaporated several times, until the odor of pyridine had disappeared. In the second method, the reaction mixture was transferred to ice water containing 1% of 6 N.sulphuric acid, and the acetate extracted with ethyl acetate or methylene chloride. The organic phase was washed twice with 6N.sulphuric acid, two or more times with 0.5 N sodium hydroxide and with water until neutral, then dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo*.

PREPARATION OF LABELLED STEROIDS

Labelled 16α-hydroxy-progesterone and 16α-hydroxy-pregnenolone were prepared by incubation of appropriate labelled substrates with micro-organisms capable of hydroxylating the steroid nucleus at the 16α position.

4-^{14}C-16α-hydroxy-progesterone (lot I)

The incubation and initial extraction were done by the CIBA Co. at Basle, Switzerland, through the courtesy of Dr. R. Neher. At the CIBA Co., 180 mg of progesterone was added to 80 uC of 4-^{14}C-progesterone (2.68 mg, Amersham). The mixture was incubated with an hydroxylating

organism (CIBA-A-7894) at 27°C for 78 hours and then extracted with ethyl acetate. The extract weighed 258 mg. Trial chromatograms done at Basle indicated that approximately 40% of the substrate had been converted to 16α-hydroxy-progesterone. The crude extract contained 1.17 x 10^8 cpm as determined in our laboratory.

The extract was divided into three parts, and each part chromatographed on an 80 g celite column using system B. In this system 16α-hydroxy-progesterone has a partition co-efficient of 0.15. The samples were dissolved in 3 ml of stationary phase, mixed with 6 g of celite and packed on the top of the column. The mobile phase was run at the rate of 60 ml per hour and collected in 10 ml fractions. Aliquots were taken for counting from each fraction from the first column and from every other fraction from the second and third columns. Fig. 1 shows the plot of radioactivity against fraction number for the first column. The fractions containing the major peak of radioactivity were combined, yielding a residue weighing 74.9 mg and containing 3.24 x 10^7 cpm.

An aliquot weighing 0.85 mg and containing 3.70 x 10^5 cpm was chromatographed for 10 hours on Whatman #1 paper, in the system toluene:propylene glycol. An autoradiograph showed seven spots, the largest and darkest of which corresponded in mobility to standard 16α-hydroxy-progesterone,

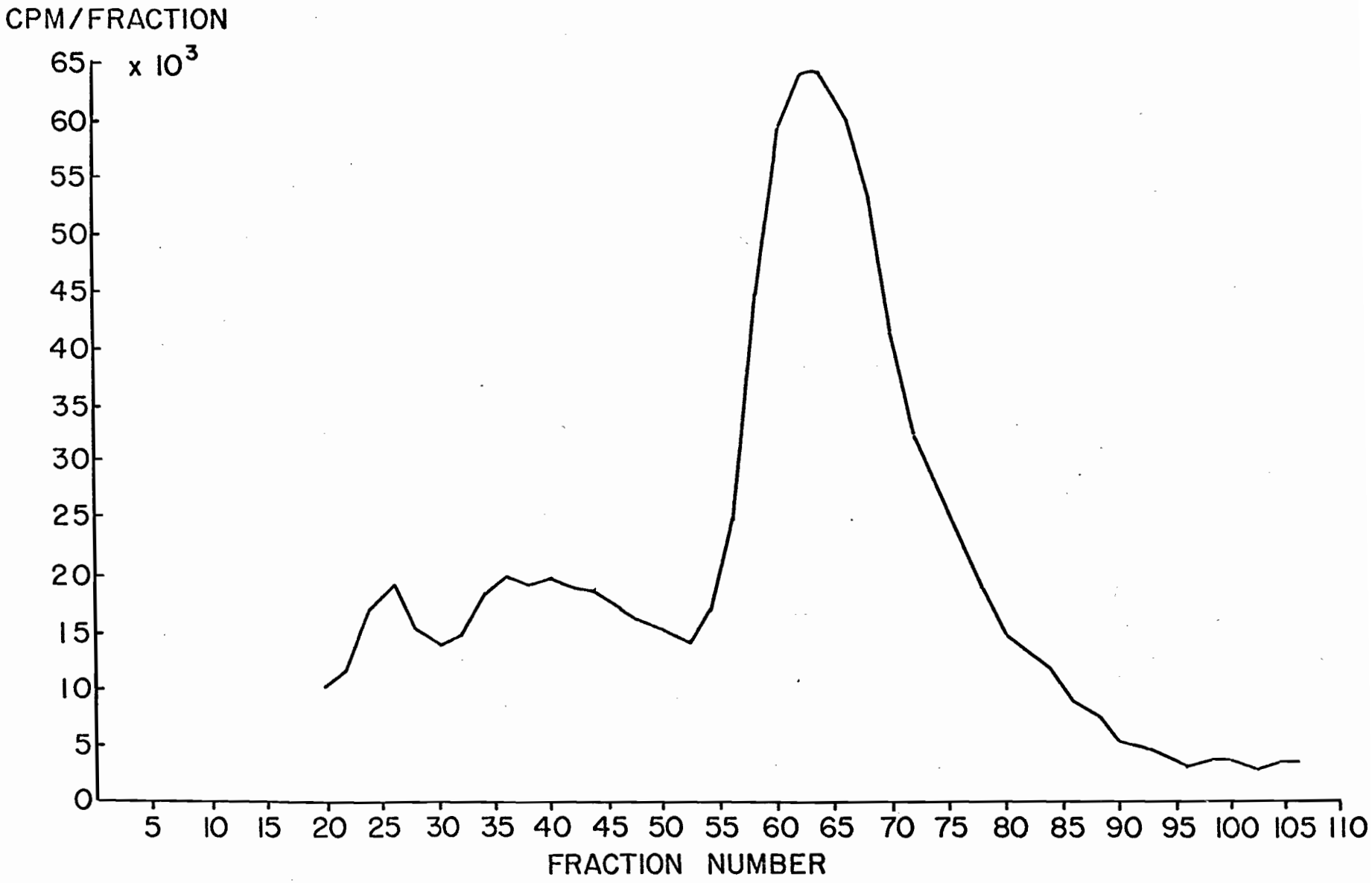

FIG. 1. 4-^{14}C-16α-HYDROXYPROGESTERONE. LOT I. FIRST CELITE COLUMN.

and to a UV-absorbing area in the extract lane. The remainder of the extract was chromatographed in the same manner. The steroid was located by its absorption of UV light and eluted with methanol, yielding a residue weighing 35.7 mg. The residue was chromatographed on a 35 g silica gel column, starting with benzene and continuing with increasing concentrations of ethyl acetate in benzene. Elution with 80% ethyl acetate gave 15.7 mg of white crystalline material. The compound was crystallized twice from ether:acetone and once from acetone:Skellysolve B, yielding 7.1 mg of coarse needles, m.p. 215-220°C (standard, 217-220°C). An infrared spectrum (KBr) was identical with that of standard 16α-hydroxy-progesterone. The specific activities of the final crystals and mother liquors (Table II) were the same, within the limits of error of measurement.

TABLE II

Crystallization of 4-^{14}C-16α-hydroxy-progesterone, lot I

	Specific activity - cpm/mg x 10^5	
	Crystals	Mother liquors
1.	6.47	
2.	6.97	6.36
3.	6.65	6.49

The final crystals were used for *in vivo* studies.

7α-^{3}H-16α-hydroxy-progesterone

This lot was prepared by incubation of 7α-^{3}H-progesterone with a strain of Streptomyces Roseochromogenus. The organism (SC-1624) was supplied by Dr. J. Fried of the Squibb Institute for Medical Research. The method of incubation was that suggested by Dr. Fried.

The organism was maintained on slants of Gould's agar. A two to four week growth was transferred to 50 ml of a liquid medium in a 250 ml Erlenmeyer flask. The medium consisted of 30 g of glucose, 20 g of soybean meal, 2.2 g of hydrogenated soybean oil and 2.5 g of $CaCO_3$, all in one litre of water. The inoculated flask was maintained at 25°C in a rotary incubator (air, 180 cycles per min., 0.5" radius). After 72 hours, a 10% transfer was made to a second flask containing 50 ml of the same medium. Tritium labelled progesterone, dissolved in 0.25 ml of dimethyl formamide, was added to the second flask, and incubated for 48 hrs. at 25°C in the rotary incubator.

The substrate, 7α-^{3}H-progesterone, was obtained from New England Nuclear Corporation, Boston, Mass., and was used as received. The supplier's label stated that the material weighed 0.016 mg and contained 500 uC of tritium. It was diluted with 7.8 mg of progesterone before use. The theoretical specific activity of the substrate was 2.81 x 10^{7} cpm per mg.

After incubation, the contents of the flask were diluted to 200 ml with water and poured into 2 litres of acetone. The mixture was filtered through glass wool and the acetone removed in vacuo. The remaining aqueous phase was diluted to 200 ml with water and extracted three times with 300 ml of methylene chloride. The latter was dried over anhydrous Na_2SO_4 and evaporated in vacuo, leaving a residue of 60.4 mg. This residue was chromatographed on thin layers in the system ethyl acetate:n-hexane::7:3. Two UV-absorbing areas were observed on the plates. The more polar of these was 3.5 cms from the origin and corresponded in mobility to standard 16α-hydroxy-progesterone. Elution of the silica gel in this area yielded a residue containing 1.66×10^8 cpm.

The residue was then chromatographed for 10 hours on Whatman #1 paper, in the system toluene:propylene glycol. Eight papers were used, each with standard 16α-hydroxy -progesterone in a separate lane. A 2 cm strip from one paper was scanned for radioactivity. The tracing showed a large symmetrical peak of radioactivity at 15 cms and a much smaller one at 9 cms. The larger peak corresponded in mobility to the standard and to a UV-absorbing area in the extract lane. The UV-absorbing areas were eluted, yielding a residue which contained 1.4×10^8 cpm. The compound was dissolved in 10% methanol in benzene for storage. As a

test of purity, a portion of the product was crystallized with a large amount of unlabelled steroid. The specific activities of the crystals and mother liquors from successive crystallizations will reflect the presence of small amounts of radioactive impurities. A portion of the product containing 1.20×10^5 cpm was mixed with 59.7 mg of unlabelled 16α-hydroxy-progesterone and the mixture crystallized from acetone:Skellysolve B, acetone:ether and acetone:Skellysolve B. The specific activities of crystals and mother liquors (Table III) were constant and equal,

TABLE III

Test of radiochemical homogeneity of 7α-^{3}H-16α-hydroxy-progesterone. Crystallization of 1.20×10^5 cpm with 59.7 mg of carrier.

	Specific activity - cpm/mg	
	Crystals	Mother liquors
1.	1967	2055
2.	2044	2090
3.	2005	1992

Predicted - 2010 cpm/mg.

within the limitations of error of measurement, and agreed well with the predicted specific activity. The product, 7α-^{3}H-16α-hydroxy-progesterone, was obtained in 70% yield. It had a calculated specific activity of 2.81×10^7 cpm per mg.

4-^{14}C-16α-hydroxy-progesterone (lot II).

The substrate, 4-^{14}C-progesterone, was obtained from New England Nuclear Corp., Boston, Mass. The vial was said to contain 0.68 mg of progesterone and 100 uc of carbon-14. A portion, containing 90 uC and 0.61 mg, was mixed with 7.14 mgms of progesterone and used as substrate.

The organism was prepared, and the incubation and extraction performed in the same manner as in the preparation of 7α-^{3}H-16α-hydroxy-progesterone. The final extract was dissolved in 15 ml of methylene chloride and to this solution was added 300 ml of methanol. A fine white precipitate appeared. The mixture was stored at 4^{o}C for 18 hours and then filtered through a sintered glass funnel. The residue remaining after evaporation of the filtrate weighed 30.9 mg and contained 1.41 x 10^{8} cpm. This residue was chromatographed on thin layers and on paper in the manner described above. The eluates from paper were chromatographed on a 1 g alumina column, starting with benzene. Elution with 2% ethanol in benzene gave 3.65 mg of a clear oil, containing 7.8 x 10^{7} cpm. A portion containing 156,000 cpm was mixed with 52.7 of 16α-hydroxy-progesterone, and crystallized three times from acetone:Skellysolve B. The specific activities of crystals and mother liquors (Table IV) were constant and equal, within the limitations of error of measurement, and agreed well with the predicted value. The product was

TABLE IV

Test of radiochemical homogeneity of 4-^{14}C-16α-hydroxy progesterone - lot II. Crystallization of 1.56×10^5 cpm with 52.7 mg of carrier.

	Specific activity - cpm/mg	
	Crystals	Mother liquors
1.	3017	2974
2.	3062	3032
3.	3055	3017

Predicted - 2960 cpm/mg.

obtained in 43% yield. Its specific activity after alumina chromatography, was 2.14×10^7 cpm/mg.

7α-^{3}H-16α-hydroxy-pregnenolone

The substrate, 7α-^{3}H-pregnenolone, was obtained from New England Nuclear Corp., Boston, Mass., and was used as received. One millicurie of tritium in 0.036 mgms of pregnenolone was mixed with 7.06 mg of unlabelled pregnenolone prior to incubation. A second strain of Streptomyces Roseochromogenus, ATCC 3347, was used in this preparation. The organism was supplied by Dr. C. Vezina, Ayerst, McKenna and Harrison, Montreal. The incubation, extraction and methanol precipitation were done in the manner described above. The residue after evaporation of the methanol filtrate weighed 36.3 mg and contained 5.13×10^8 cpm. This residue was divided into four parts and chromato-

graphed on thin layers in the system ethyl acetate:n-hexane ::2:1. Standard pregnenolone and 16α-hydroxy-pregnenolone were run beside the extracts and located by spraying with phosphomolybdic acid. The area corresponding to the 16α-hydroxy-pregnenolone standard was eluted, yielding 1.65 mg of crystalline residue containing 1.00×10^8 cpm. This material was chromatographed for 32 hrs. on two strips of Whatman #1 paper, in the system toluene:propylene glycol. A scan of the extract lane revealed a single peak of radioactivity corresponding in mobility to standard 16α-hydroxy-pregnenolone. The area containing radioactivity was eluted, yielding a residue which contained 7.24×10^7 cpm. A portion containing 1.45×10^6 cpm was mixed with 50.4 mg of 16α-hydroxy-pregnenolone. The mixture was crystallized from methanol:ethyl acetate, acetone:methanol and methanol. Because of the insolubility of the compound in toluene phosphor, it was necessary to count small amounts of crystals and mother liquors. Crystals and mother liquors were weighed accurately and dissolved in 100 mls of methanol. Aliquots of these dilutions containing 50 to 100 ug were used for counting. The specific activities of crystals and mother liquors (Table V) were constant and agreed well with the predicted specific activity, within the limitations of error of measurement. The product was obtained in 8% yield. It had a calculated specific of 1.28×10^8 cpm/mg.

TABLE V

Test of radiochemical homogeneity of 7α-^{3}H-16α-hydroxy pregnenolone. Crystallization of 1.45 x 10^{6} cpm with 50.4 mg of carrier.

	Specific activity - cpm/mg x 10^{4}	
	Crystals	Mother liquors
1.	2.95	2.95
2.	3.04	2.97
3.	3.02	2.90

Predicted - 2.87 x 10^{4} cpm/mg.

Purification of 4-^{14}C-progesterone

The labelled progesterone was obtained from New England Nuclear Corp., Boston, Mass. Approximately 10 uC, (0.07 mg) was chromatographed for 2 hrs. on Whatman #1 paper in the system n-heptane:propylene glycol. A single peak of radioactivity was found. Elution of the area containing radioactivity yielded 3.4 mg of residue containing 2.1 x 10^{7} cpm. The residue was mixed with 5.2 mg of progesterone and chromatographed on a 1 g alumina column, starting with n-hexane:benzene::4:6. Elution with n-hexane:benzene::2:8 and with benzene gave 4.5 mg of colorless crystalline material containing 1.6 x 10^{7} cpm. The residue was crystallized from ether:Skellysolve B. The specific activities of crystals and mother liquors were constant within the limitations of error of measurement (Table VI). The

specific activity was 3.65×10^6 cpm per mg.

TABLE VI

Crystallization of 4-^{14}C-progesterone

	Specific activity - cpm/ug	
	Crystals	Mother liquors
1.	3651	3447

Predicted - 3533 cpm/ug.

PREPARATION OF ISOPREGNANOLONE

Isopregnanolone was prepared by isomerization of 3α-hydroxy-5β-pregnan-20-one (50). A solution of 201 mg of 3α-hydroxy-5β-pregnan-20-one in 30 mls of 5% methanolic KOH was refluxed for 5 hours. The solution was cooled to room temperature, diluted to 300 mls with water and extracted with ethyl acetate, 1 x 300 mls and 2 x 150 mls. The ethyl acetate was washed with water, 3 x 25 mls, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* at 40°C. The residue was chromatographed on a 20 g alumina column, starting with n-hexane:methylene chloride::1:1, and continuing with increasing concentrations of methylene chloride. Elution with the starting solvent yielded 151 mgms of crystalline compound which proved to be identical with the starting material. Elution of the column with methylene chloride

yielded 40.8 mg of crystalline residue. Crystallization from ether:n-heptane gave 31.2 mg of fine needles, m.p. 147-149°C, reported 145-147°C (40). An infrared spectrum (CS_2) was identical with that of an authentic sample of isopregnanolone generously provided by Dr. David Fukushima. The overall yield of isopregnanolone was 15%.

A second batch of isopregnanolone was made in the same way, starting with 1.01 g of 3α-hydroxy-5β-pregnan-20-one. The reflux was maintained for seven hours. The yield of isopregnanolone was 12%.

PREPARATION OF ISOPREGNANOLONE ACETATE

Isopregnanolone, 96 mg, was acetylated in 0.4 ml of pyridine and 0.2 ml of acetic anhydride. The solvents were evaporated under nitrogen, and the residue chromatographed on a 15 g alumina column, starting with Skellysolve B. Elution with 20% benzene in Skellysolve B yielded 103 mg of residue, which on crystallization from acetone:n-heptane gave 78.3 mg of fine needles, m.p. 162-165°C. An infrared spectrum (CS_2) showed no absorption due to free hydroxyl groups. There were bands at 1730 Cm^{-1} and 1240 Cm^{-1} indicative of an acetate group. We were not able to compare the spectrum with that of an otherwise authenticated standard.

PREPARATION OF 16α-HYDROXY-PROGESTERONE ACETATE

One gram of 16α-hydroxy-progesterone was acetylated in 6 mls of pyridine and 3 mls of acetic anhydride. The reaction was stopped with 200 mls of water containing 1% of 6 N sulphuric acid. The residue after extraction of the water was crystallized from ether, yielding 863 mg of large needles, m.p. 135-136.5^{o}C. An infrared spectrum (CS_2) showed no evidence of free hydroxyl groups. A spectrum in KBr showed bands at 1735 cms^{-1} and 1245 cm^{-1}, not present in the spectrum of the starting material, and ascribed to an acetate group. Bands at 1620, 1675 and 1705 cms^{-1} indicated the retention of the Δ^4-3-ketone and the 20-ketone. We were not able to compare the spectrum with one of otherwise authenticated material.

PREPARATION OF 16α,20β-DIHYDROXY,-Δ^4-PREGNEN-3-ONE, 16-ACETATE

This compound was prepared from 16α-hydroxy-progesterone acetate by reduction of the two ketones, and subsequent oxidation of the allylic hydroxyl group at C3.

Reduction with $NaBH_4$ was done by the method of Norymberski and Wood (52). A solution of 30.5 mg of 16 - hydroxy-progesterone acetate in 6 mls of methanol was cooled to 0^{o}C and treated with 4.9 mg of $NaBH_4$. After one hour at 0^{o}C, the reaction was stopped with 6 drops of glacial acetic acid, and the methanol evaporated. The residue was dissolved

in ethyl acetate, which was then washed with 0.1 N NaOH and water, dried over Na_2SO_4, filtered and evaporated in vacuo at 40°C. Oxidation with dichloro-dicyano-benzoquinone was performed by the method of Burn et al (53), with minor modifications. The products of reduction were dissolved in 2 ml of freshly distilled dioxane and treated with 22.2 mg of dichloro-dicyano-benzoquinone. The reaction mixture was left in the dark at room temperature for 24 hours, then filtered and evaporated to dryness. The reaction products were dissolved in ethyl acetate, washed with 0.5 N NaOH and water, dried over Na_2SO_4, filtered and evaporated to dryness in vacuo. The residue, 32.6 mg, was chromatographed on a 5 g silica gel column, starting with 1% ethanol in benzene. Elution with 3% ethanol in benzene gave 22.6 mg of yellowish non-crystalline material. Crystallization from methanol: ether:Skellysolve B yielded 13.2 mg of small coarse needles, m.p. 173-177°C. An infrared spectrum (KBr) showed major absorption bands at 1730, 1665, 1615, 1245 and 1085 cm^{-1}. There was no absorption at 1700 cm^{-1}. We were not able to compare the synthesized compound with an authenticated standard. However, it is known (52) that the major product of reduction of the 20-ketone with $NaBH_4$ is the 20β-hydroxy group. Also, under the conditions employed, only α,β-unsaturated alcohols are oxidized by dichloro-dicyano-benzoquinone. The infrared spectrum indicates the

disappearance of the 20-ketone, the retention of the α,β unsaturated ketone and the acetate group, and the appearance of an hydroxyl group. With this evidence we can with some confidence assign to the product the structure 16α,20β-dihydroxy-Δ^4-pregnen-3-one, 16-acetate.

PREPARATION OF 16α-HYDROXY-PREGNENOLONE

One gram of 16α-hydroxy-pregnenolone,3-acetate, was saponified by the method of Ringold (51). The acetate was dissolved in 80 mls of cold methanol and added to 20 mls of cold 5% methanolic potassium hydroxide. After 90 mins. the steroid was precipitated by the addition of water, and collected on a sintered glass filter. The residue weighed 860 mg. Crystallization from acetone:methanol gave 459 mg of fine needles, m.p. 230-232°C, standard 229-232°C. An infrared spectrum (KBr) was identical to that of standard 16α-hydroxy-pregnenolone.

STANDARDIZATION OF 1-^{14}C-ACETIC ANHYDRIDE

The stock solution of 1-^{14}C-acetic anhydride, specific activity 10 mC. per mM., was obtained from New England Nuclear Corporation. One mC. (10.2 mg) in 0.04 ml of benzene was diluted with 0.22 ml of acetic anhydride and 2.0 mls of dry benzene to give solution 1. Serial 10-fold

dilutions with 10% acetic anhydride in benzene gave solutions 2 and 3. The specific activity of the acetic anhydride in each solution was determined by acetylation of desoxycorticosterone, and measurement of the specific activity of the resultant desoxycorticosterone acetate.

Solution #1

Thirty-five mg of desoxycorticosterone was dissolved in 0.2 ml of pyridine and 0.3 ml of solution #1 in a glass stoppered tube, and stored in the dark at 37°C for 17 hours. The solvents were evaporated under nitrogen. The residue, 40.2 mg, was chromatographed on a 5 g silica gel column, starting with benzene. Elution with 2% ethanol in benzene yielded 39.4 mg of crystalline residue. The acetate was crystallized three times from acetone:Skellysolve B. The specific activities (Table VII) of crystals and mother liquors were constant after the first crystallization.

Solutions #2 and #3

Solutions #2 and #3 were standardized in the same way. The specific activities of crystals and mother liquors are shown in Table VII.

The labelled acetic anhydride was used to determine the specific activities of certain urinary metabolites in Experiments 3 and 4. The isolated compound was acetylated with one of the standardized solutions of 1-^{14}C-Acetic anhydride, mixed with carrier and crystallized to constant

TABLE VII

Standardization of 1-^{14}C-Acetic Anhydride

		Specific Activity - cpm/mg DOCA	
		Crystals	Mother liquors
Sol'n I	1.	3.13×10^5	3.72×10^5
	2.	3.15	3.12
	3.	3.20	3.20
Sol'n II	1.	3.14×10^4	3.03×10^4
	2.	3.13	2.69
	3.	3.16	3.24
	4.	3.23	3.26
Sol'n III	1.	3.47×10^3	3.43×10^3
	2.	3.52	3.55
	3.	3.47	3.46

specific activity. The specific activity of the isolated steroid was calculated according to the expression

$$S.A. = a \times \frac{b}{c} \times d$$

where

S.A. = specific activity of the isolated metabolite, cpm/mg.

a = S.A. of DOC-^{14}C-acetate, cpm/mg.

b = molecular wt. of DOCA.

c = molecular wt. of the metabolite.

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

EXPERIMENTAL

EXPERIMENT #1: METABOLISM OF 16α-HYDROXY-PROGESTERONE

This study was designed to enable us to isolate large amounts of urinary metabolites of 16α-hydroxy-progesterone. The subject, a 36 year old normal male, was given an intravenous injection of 4-^{14}C-16α-hydroxy-progesterone (lot #1), weighing 2 mg and containing 1.3 x 10^6 cpm. Urine was collected for 4 days (days 1-4) and frozen. At a later date the subject was given 1500 mg of 16α-hydroxy-progesterone per os, in 100 mg doses over 48 hours. Urine was collected for three days (days 5-7).

The individual 24 hour urine collections were extracted by the procedure of Edwards, Kellie and Wade (54). Ammonium sulphate was added to the urine to give a concentration of 50% (w/v). The urine was extracted three times with one-half volumes of ether:ethanol (3:1). The organic phase was filtered and evaporated *in vacuo* at 40°C. Absolute ethanol was added during evaporation to aid in the removal of water. Finally the residue was dissolved in ethanol, the solution filtered and the solvent evaporated *in vacuo*. The extracts of days 5 to 7 were pooled, the extracts of days 1 to 4 kept separately.

Steroid conjugates were hydrolyzed with Glusulase. The extracts of days 1 to 4 were dissolved in 160 mls of 0.5M sodium acetate buffer, pH 5.2, treated with 2 ml of Glusulase and incubated at 37°C for 60 to 80 hrs. The pooled extracts of days 5 to 7 were similarly treated, using 500 mls of buffer, 6 mls of Glusulase, and an 18 hr. incubation period. Following incubation, sodium chloride, 20% (w/v), was added to the hydrolysates and a neutral ethyl acetate extract obtained. All extracts were oily, and a dark purple color. Aliquots of urines, hydrolysates and neutral extracts of days 1 to 4 were taken for counting (Table VIII).

TABLE VIII

Experiment 1. Injection of 4-^{14}C-16α hydroxy progesterone, 1.3×10^6 cpm.

Radioactivity recovered - cpm x 10^5

	Urine	Hydrolysate	Neutral extract
Day I	3.02	2.71	2.50
II	1.10	0.98	0.85
III	0.60	0.54	0.39
IV	0.30	0.27	0.20
Total	5.02	4.50	3.94
% Dose	39%		30%

The combined neutral extract of the seven days urine weighed 2.74 g and contained 395,000 cpm. It was chromato-

graphed on a 300 g silica gel column, starting with n-hexane and continuing with the solvent series n-hexane, benzene, ether and ethyl acetate. Fractions of 200 mls were collected. Considerable difficulty was encountered with this column, and no useful fractionation of radioactivity was achieved. All fractions containing radioactivity were pooled. The residue weighed 1.74 g and contained all of the radioactivity.

The extract was re-chromatographed on a 100 g silica gel column, using methylene chloride with increasing concentrations of ethanol. Fractions of 100 mls were collected and aliquots removed for measurement of radioactivity (Fig. 2). Two peaks of radioactivity were found. The first, peak A, eluted with 2 and 4% ethanol, weighed 750 mg and contained 112,000 cpm. The second, peak B, eluted with 8 and 12% ethanol, weighed 420 mg and contained 177,000 cpm.

The residue of pool A was chromatographed on thin-layers in the system ethyl acetate:n-hexane::2:1. Elution and counting of 1 cm strips from trial chromatograms showed that the bulk of the radioactivity was in an area between a line 6 cms from the origin and a distinctive band of red pigment at about 13 cms. The extract was chromatographed on 30 thin layers. Elution yielded an oily purple residue weighing 333 mg and containing 67,300 cpm. This residue was chromatographed on a 130 g celite column in system A.

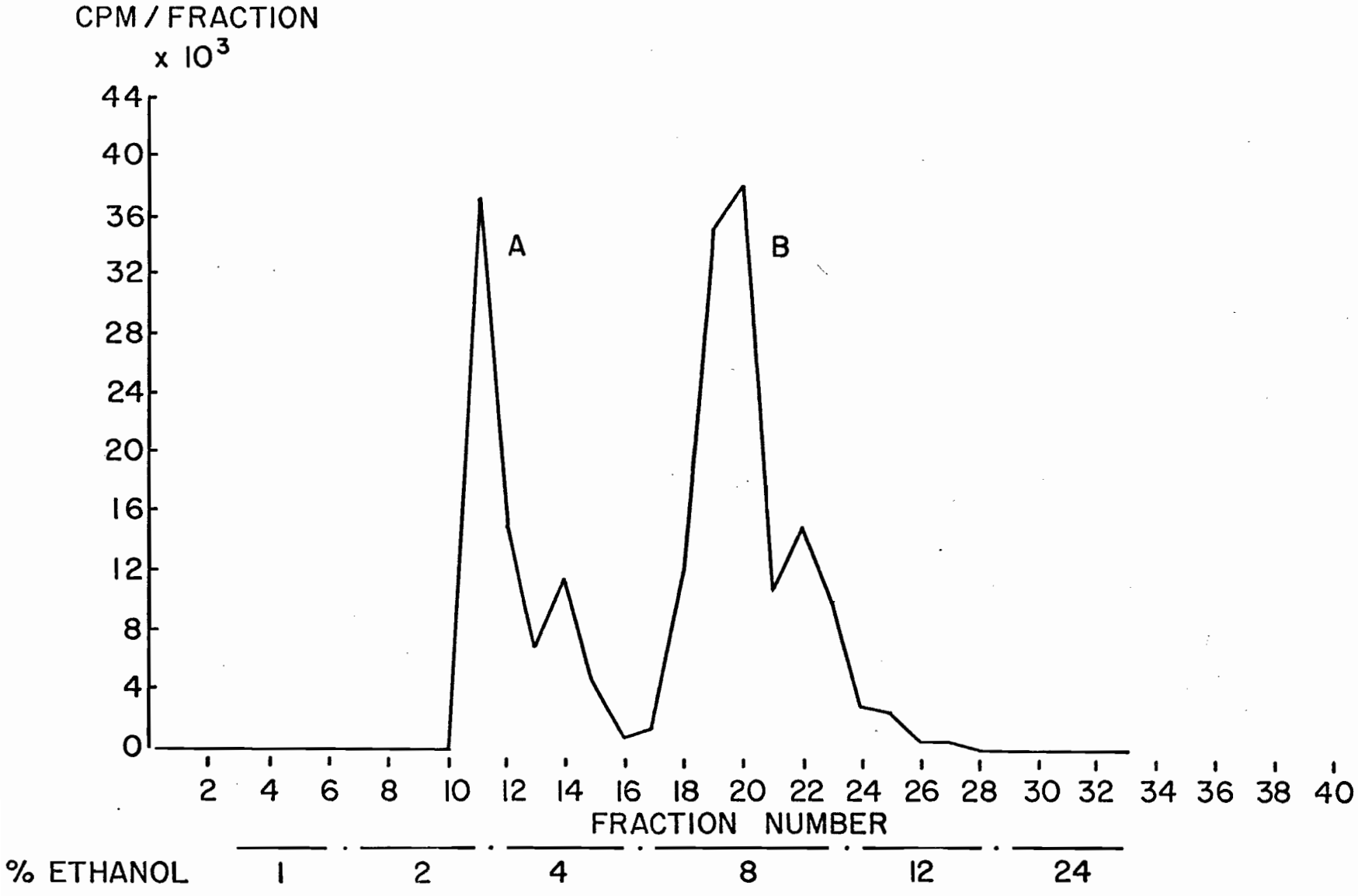

FIG. 2. 100 GM SILICA GEL COLUMN. EXPERIMENT 1.

The sample was dissolved in 5 mls of stationary phase, mixed with 10 g of celite and packed on the top of the column. Mobile phase was collected in 10 ml fractions, at the rate of 30 mls per hr. Aliquots were taken from every third tube for measurement of radioactivity (Fig. 3). Fractions 55 to 100, representing the major peak, were pooled and evaporated, leaving an oily purple residue weighing 43 mg and containing 42,100 cpm. The residue was chromatographed for 10 hrs. on Whatman #1 paper, in the system n-heptane: propylene glycol. Twelve papers were used, each with standard isopregnanolone in a separate lane. The standard was located with phosphomolybdic acid. The area of the extract lane opposite the standard was eluted, yielding 24.4 mg and 20,500 cpm. This material was further purified by chromatography on a 2 g silica gel column, using the solvent series: n-hexane, benzene and ether. Elution with benzene: ether::1:1 and ether gave 15.4 mg of clear oil containing 20,500 cpm. Crystallization from ether:Skellysolve B yielded 7.3 mg of fine needles, m.p. 147-148°C, m.m.p. 147-148°C, standard m.p. 147-149°C. An infrared spectrum (CS_2) was identical with that of standard isoprenanolone. The specific activity of the compound was 1.4×10^3 cpm per mg.

The residue of pool B from the silica gel column was chromatographed on thin-layers in the system chloroform: methanol:water::90:10:1. Trial chromatograms demonstrated

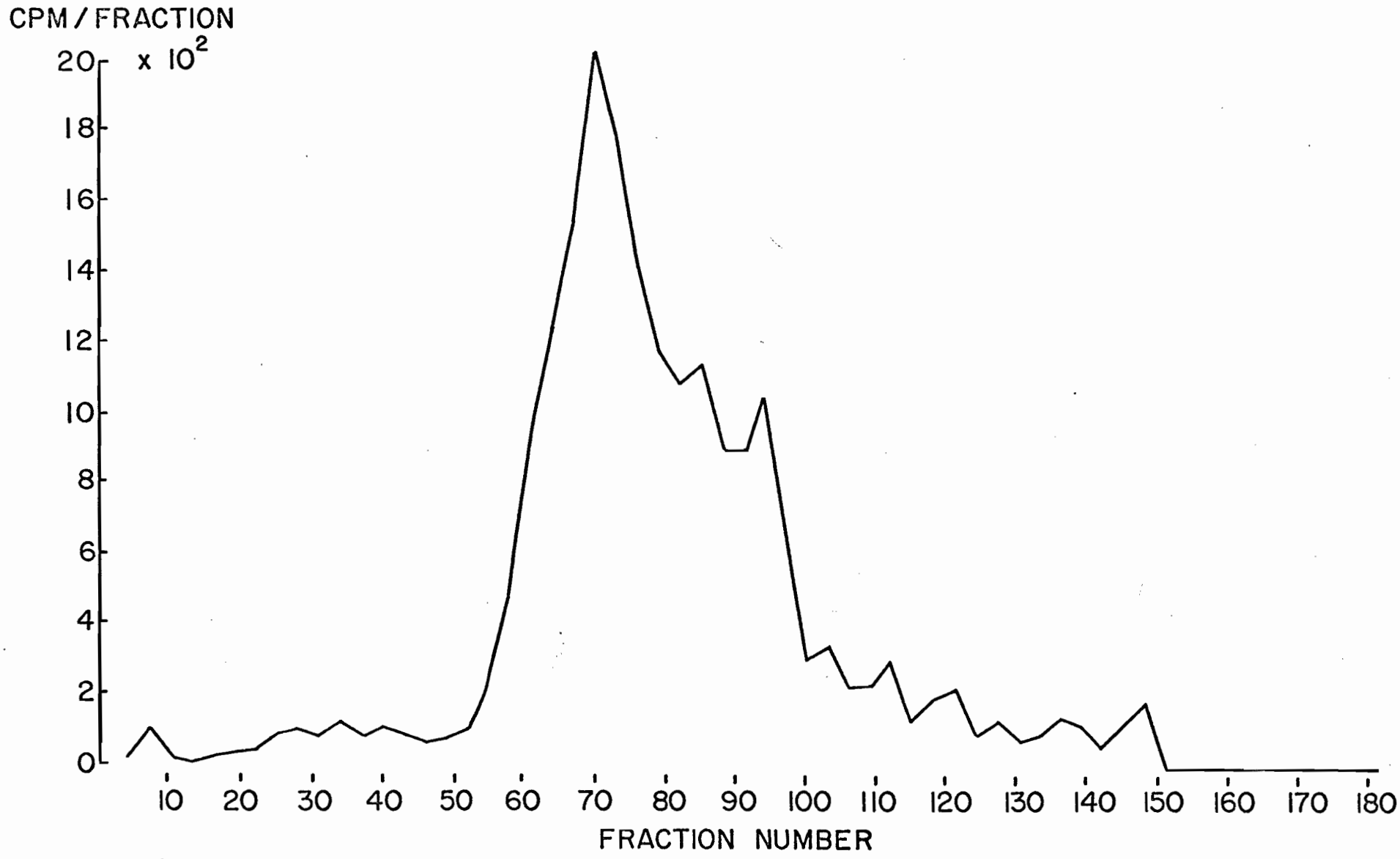

FIG. 3. EXPERIMENT I. CELITE COLUMN. n-HEXANE : METHANOL : WATER. 100:90:10. FRACTION A.

that most of the radioactivity had a mobility between that of cortisol and that of 16α-hydroxy-progesterone. Equal portions of extract were applied to each of 20 thin-layers. Development and elution yielded 158 mg of highly colored oil, containing 117,000 cpm. The residue was further fractionated on a 150 g celite column in system D. The column was prepared with 1 ml of stationary phase per g of celite. The sample was packed on the column, using 10 ml of stationary phase and 10 g of celite. Mobile phase was collected in 10 ml fractions, at the rate of 20 mls per hour. Aliquots were taken from every second tube for measurement of radioactivity (Fig. 4). Two major peaks of radioactivity were found. The first, peak B1, was eluted in fractions 80 to 125. Pooling and evaporation of solvents left a residue which weighed 31.1 mg and contained 26,100 cpm. The residue from tubes 126 to 198, peak B2, weighed 36.1 mg and contained 69,700 cpm.

Fraction B1 was chromatographed for 40 hours on Whatman #1 paper in the system toluene:propylene glycol. Equal portions were applied to each of six papers. Scanning of the papers showed two peaks of radioactivity, the first at an average distance of 16 cms, the second at 27 cms. Elution of the paper corresponding to the first peak, B3, yielded 14.4 mg of oil, containing 8,200 cpm. The second peak, B4, yielded 21.4 mg and 10,600 cpm.

FIG. 4. EXPERIMENT I. CELITE COLUMN - BENZENE: CYCLOHEXANE: METHANOL: WATER. 1:2:3:3
FRACTION B.

The residue B3 was chromatographed on a 2 g silica gel column, starting with benzene. Elution with 8% ethanol in benzene gave 9.1 mg of colorless oil, which on crystallization from acetone:Skellysolve B yielded 4.6 mg of fine needles, m.p. 230-232°C. An infrared spectrum (KBr) showed no evidence of absorption by carbonyl groups. Three mg of the compound was acetylated. An infrared spectrum of the acetate (CS_2) was identical with that of a sample of 5α-pregnan-3α,16α,20α-triol, triacetate, generously donated by Dr. Hans Hirschmann. The melting point of the free compound, 230-232°C, agrees with the reported melting point, 230-232°C (15). The specific activity of the free compound was 1.3×10^3 cpm/mg.

Fraction B4 from paper was chromatographed on a 2 g silica gel column, starting with benzene. Elution with 4 and 8%, ethanol in benzene gave 9.2 mg of colorless oil. An infrared spectrum (CS_2) was identical with that of a sample of 3α,16α-dihydroxy-5α-pregnan-20-one obtained from Dr. R. Neher. Crystallization from acetone:Skellysolve B gave 4.6 mg of fine needles, m.p. 209-211°C., m.m.p. 208-210°C, standard, 210-211°C. The specific activity of the compound was 1.1×10^3 cpm per mg.

Fraction B2, from the celite column, was chromatographed for 55 hours on Whatman #1 paper in the system toluene:propylene glycol. Equal portions were applied to

each of 6 papers. Scanning for radioactivity revealed two peaks, at average distances of 15 and 25 cms. The area corresponding to the first peak, B5, yielded 13.8 mg of yellow oil. The second area, B6, gave 19.1 mg of colorless oil.

The residue B5 was chromatographed on a 1 g silica gel column, starting with benzene. Elution with 6 and 8% ethanol in benzene gave 7.9 mg of a colorless glassy compound, which on crystallization from acetone:Skellysolve B gave 3.7 mg of fine needles, m.p. 210-215°C. An infrared spectrum (KBr) was identical with that of a sample of 5β-pregnan-3α,16α,20α-triol, generously provided by Dr. Hans Hirschmann. Recrystallization from acetone:methanol and acetone gave 0.53 mg of fine needles, m.p. 224-226°C, m.m.p. 227-228°C, standard, 227-228°C. The specific activity of the crystals was 2.4×10^3 cpm per mg.

The second, less polar fraction from the paper chromatogram, B6, was crystallized from acetone:Skellysolve B to give 9.4 mg of fine needles, m.p. 211-212°C. Three mg of the crystals were acetylated. An infrared spectrum (CS_2) was identical to that of a sample of 3α,16α-dihydroxy-5β-pregnan-20-one, 3,16-diacetate. The latter compound was the gift of Dr. Hans Hirschmann. The diacetate was crystallized from acetone:methanol, yielding 1.9 mg of crystals, m.p. 129-130°C, m.m.p. 129-130°C, standard, m.p. 129-130°C. The

melting point of the free compound agrees well with the reported melting point of 209-212^{o}C (14). Its specific activity was 2.8 x 10^{3} cpm per mg.

EXPERIMENT 2: METABOLISM OF 16$_\alpha$-HYDROXY-PROGESTERONE

The object of this study was to confirm the findings of Experiment 1. The methods of isolation, however, were modified. The subject, a 32 year old normal male, was given an intravenous injection of 7α-^{3}H-16α-hydroxy-progesterone (2.98×10^6 cpm), and an oral load of 1 g of 16α-hydroxy-progesterone, in 100 mg doses over a period of 2 days. The oral feeding began at the time of injection. Urine was collected for 4 days, each day's collection being kept separate.

Steroid conjugates were hydrolyzed with Glusulase, 1 ml per 100 ml of urine. The urine was incubated for 5 days at 37°C. On the third day additional Glusulase, 0.5 ml percent, was added. Following incubation, neutral ethyl acetate extracts were obtained. Aliquots for measurement of radioactivity were taken from the urine and neutral extracts for each day (Table IX). The counts were not corrected for quenching.

The combined neutral extract weighed 1057 mg and contained 1.34×10^6 cpm. It was chromatographed on a 250 g silica gel column, starting with 1% ethanol in methylene chloride. The chromatogram was developed with increasing concentrations of ethanol in methylene chloride, at a rate of 30 to 40 mls per hour. Individual fractions varied from 8 to 11 mls. The fractions were pooled in

TABLE IX

EXPERIMENT 2. Injection of 7α-^{3}H-16α-hydroxy progesterone, 2.98 x 10^{6} cpm.

Radioactivity recovered - cpm x 10^{5}

	Urine	Neutral extract
Day I	8.86	7.94
II	4.18	3.71
III	1.57	1.05
IV	0.83	0.68
Total	15.44	13.38
% Dose	52%	44%

groups of four and assayed for radioactivity. Five definite peaks were found (Fig. 5), and fractions were pooled accordingly. The origin, weight and radioactivity of each residue is shown in Table X.

The residue of pool II was chromatographed on a 20 g celite column, in system D. The sample was dissolved in mobile phase for application to the column. Mobile phase was collected in 6 ml fractions at a rate of 24 mls per hr. Aliquots were taken from each fraction for counting. A single peak of radioactivity was found (Fig. 6) and fractions were pooled accordingly. The residue weighed 38.2 mg and contained 31,000 cpm. It was chromatographed for 6 hours on 6 strips of Whatman #1 paper in the system toluene:propylene glycol. Radioactivity was located by elution and counting of 2 cm strips from origin to end of paper. A single peak was

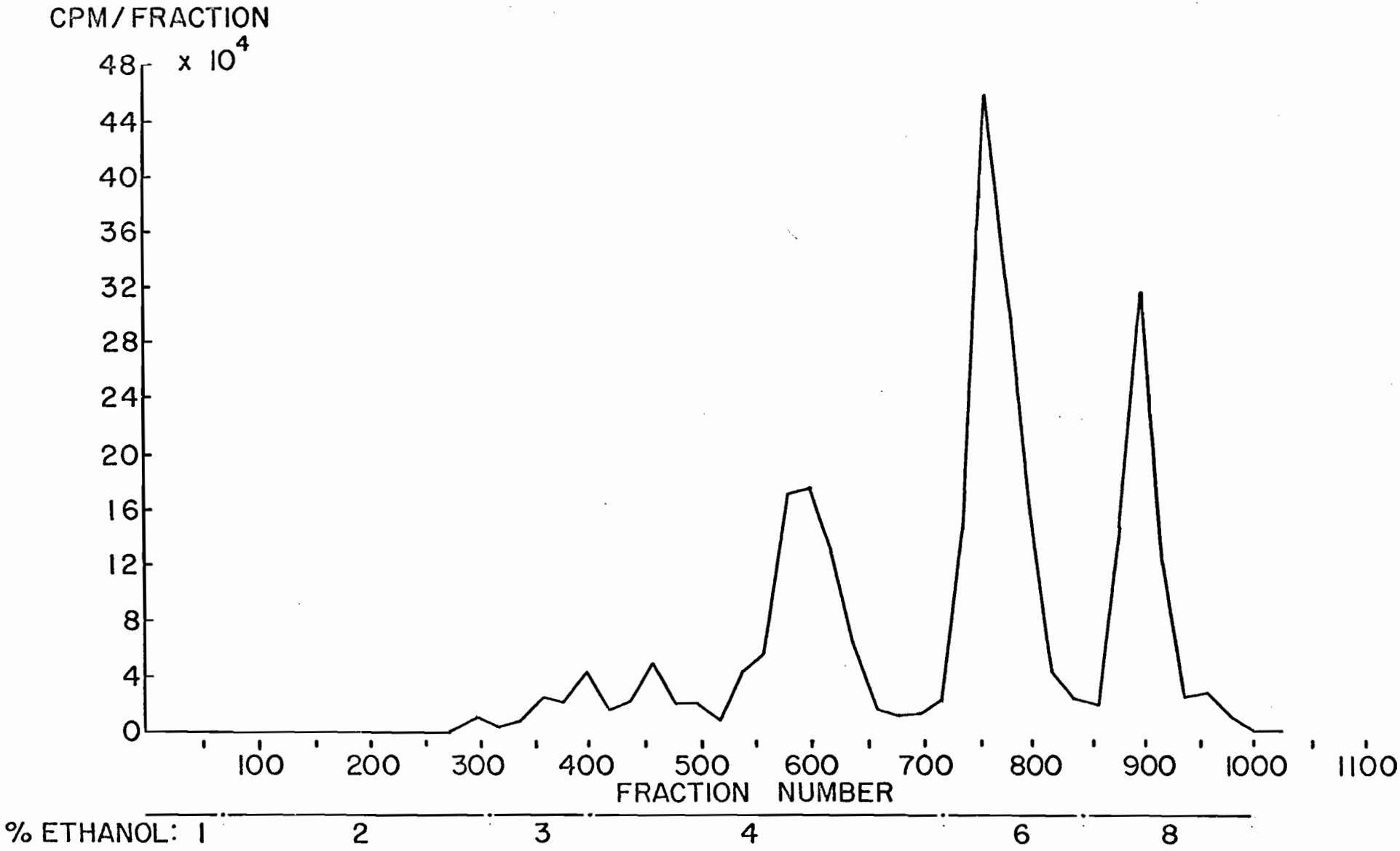

FIG.5. EXPERIMENT 2. SILICA GEL COLUMN CHROMATOGRAPHY OF NEUTRAL EXTRACT.

TABLE X

EXPERIMENT 2. Residues from initial silica gel column.

% Ethanol	Fraction no.	Pool	
1%	1		
	60		Discarded
2%	293		
	309	I	26.7 mg, 6000 cpm
3%	372		
	405	II	78.9 mg, 33,700 cpm
	432		
	472	III	30.8 mg, 32,000 cpm
	532	IV	19.2 mg, 7,700 cpm
4%	660	V	64.9 mg, 236,500 cpm
	708	VI	9.8 mg, 4,100 cpm
	718	VII	154 mg, 368,700 cpm
6%	824		
	841	VIII	14.2 mg, 8,900 cpm
	864		
8%	940	IX	63.2 mg, 237,000 cpm
	988	X	33.6 mg, 7,300 cpm
10%	1084		
20%	1 litre	XI	42.2 mg, 22,000 cpm

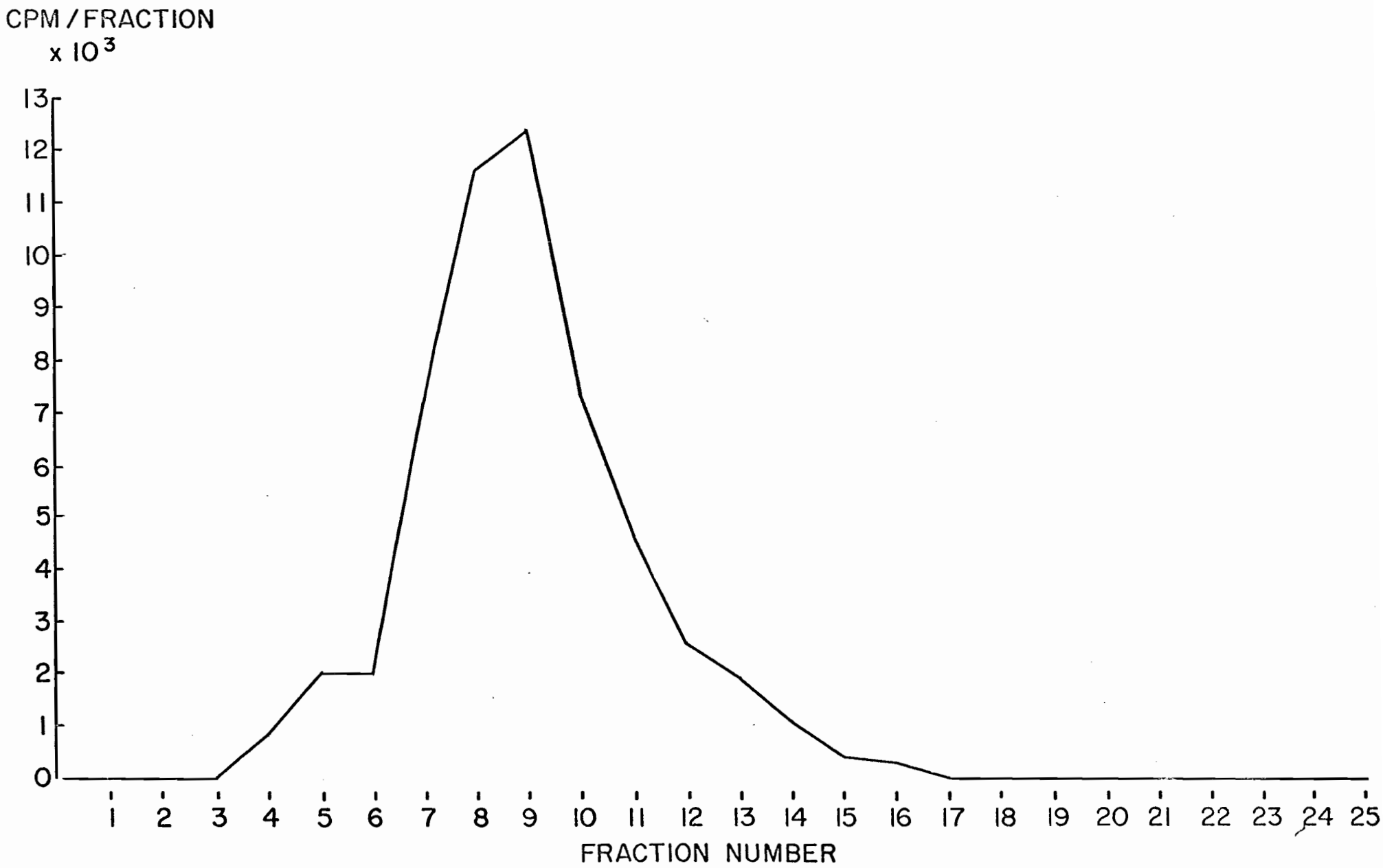

FIG.6. CELITE COLUMN CHROMATOGRAM - RESIDUE OF POOL II, EXPERIMENT 2.
SYSTEM: BENZENE: CYCLOHEXANE: METHANOL: WATER. 1:2:3:3

found on each paper at an average distance of 10 cms. The residue from the radioactive eluates weighed 11.2 mg and contained 23,000 cpm. This residue was further purified on a 1 g silica gel column, using the solvent series: n-hexane, benzene, ether and ethyl acetate. The column was started with n-hexane:benzene::1:1. Elution with ether: ethyl acetate::80:20 gave 3.9 mg of colorless oil containing 15,700 cpm. Crystallization from acetone:Skellysolve B yielded 1.6 mg of fine needles, m.p. 205-206^{o}C. A well defined infrared spectrum (KBr) was obtained but the compound remains unidentified.

The residue of pool III was chromatographed for 9 hours on Whatman #1 paper, in the system toluene propylene glycol. Equal portions were applied to each of 6 papers. Radioactivity was located by elution and counting of 2 cms strips from origin to the end of the paper. A single peak of radioactivity was found on each paper, at an average distance of 16 cms. The residue from the eluates containing radioactivity weighed 13.5 mg and contained 26,300 cpm. It was chromatographed on a 2 g alumina column, starting with n-hexane:benzene::1:1 and continuing with increasing concentrations of benzene in n-hexane, and of ethanol in benzene. Elution with 2% ethanol in benzene yielded a colorless glassy compound weighing 6.3 mg and containing 24,500 cpm. Crystallization from acetone:Skellysolve B

with a trace of methanol gave 4.1 mg of coarse needles. An infrared spectrum (KBr) was identical with that of 16α-hydroxy-progesterone. Recrystallization from acetone gave 2.3 mg of fine needles, m.p. 221-222°C, m.m.p. 220-222°C, standard m.p. 220-222°C. The compound had a specific activity of 5.2×10^3 cpm per mg.

The residue of pool V was chromatographed for 24 hrs. on Whatman #3 MM paper, in the system toluene:propylene glycol. Equal portions were applied to each of 6 papers. Scanning for radioactivity demonstrated a single peak on each paper at an average distance of 13 cms. Elution of the areas containing radioactivity yielded 33.7 mg of yellow oily material containing 182,000 cpm. This residue was chromatographed on a 5 g silica gel column, starting with benzene. Elution with 4 and 6% ethanol in benzene yielded 25.7 mg of colorless oil, containing 195,000 cpm. Crystallization from acetone gave 18.2 mg of fine needles. Unfortunately, these crystals were lost. The mother liquors from the first crystallization were dried, and the residue crystallized from acetone:methanol, yielding 2.9 mg of fine needles, m.p. 205-212°C. An infrared spectrum was identical with that of 3α,16α-dihydroxy-5α-pregnan-20-one. Recrystallization from acetone:Skellysolve B gave 1.4 mg of fine needles, m.p. 208-211, m.m.p. 209-211, standard m.p. 209-211. Its specific activity was 5.4×10^3 cpm per mg.

A portion of the residue of pool VII, weighing 51 mg and containing 124,000 cpm was chromatographed on a 10 g silica gel column, starting with benzene. Elution with 6% ethanol in benzene gave 42.0 mg of yellow glassy material containing all of the radioactivity. It was then chromatographed on a 50 g celite column, in system D. The sample was packed on the column, using 2.5 ml of stationary phase and 5 g of celite. Mobile phase was collected in 10 ml fractions, at a rate of 20 mls per hr. Fractions were pooled in groups of 2 and aliquots taken for counting. Two peaks of radioactivity were found (Fig. 7). Fractions 31-48 yielded 17.0 mg of colorless oil containing 80,500 cpm. Crystallization from acetone:Skellysolve B with a trace of methanol gave 9.8 mg of fine needles, m.p. 211-212°C, specific activity 6.2×10^3 cpm per mg. An infrared spectrum (KBr) was identical with that of 3α,16α-dihydroxy-5β-pregnan-20-one isolated in Experiment 1. A mixed melting point (fraction B6, Expt. 1) was 211-212°C, reported m.p. 209-212°C (14).

The residue of pool IX was chromatographed for 65 hours on Whatman #3 MM paper, in the system toluene:propylene glycol. Equal portions were applied to 10 cm lines on each of six papers. A single peak of radioactivity was found on each paper, at an average distance of 20 cms. Elution of the papers yielded 42.7 mg of yellow oil, containing 237,000 cpm.

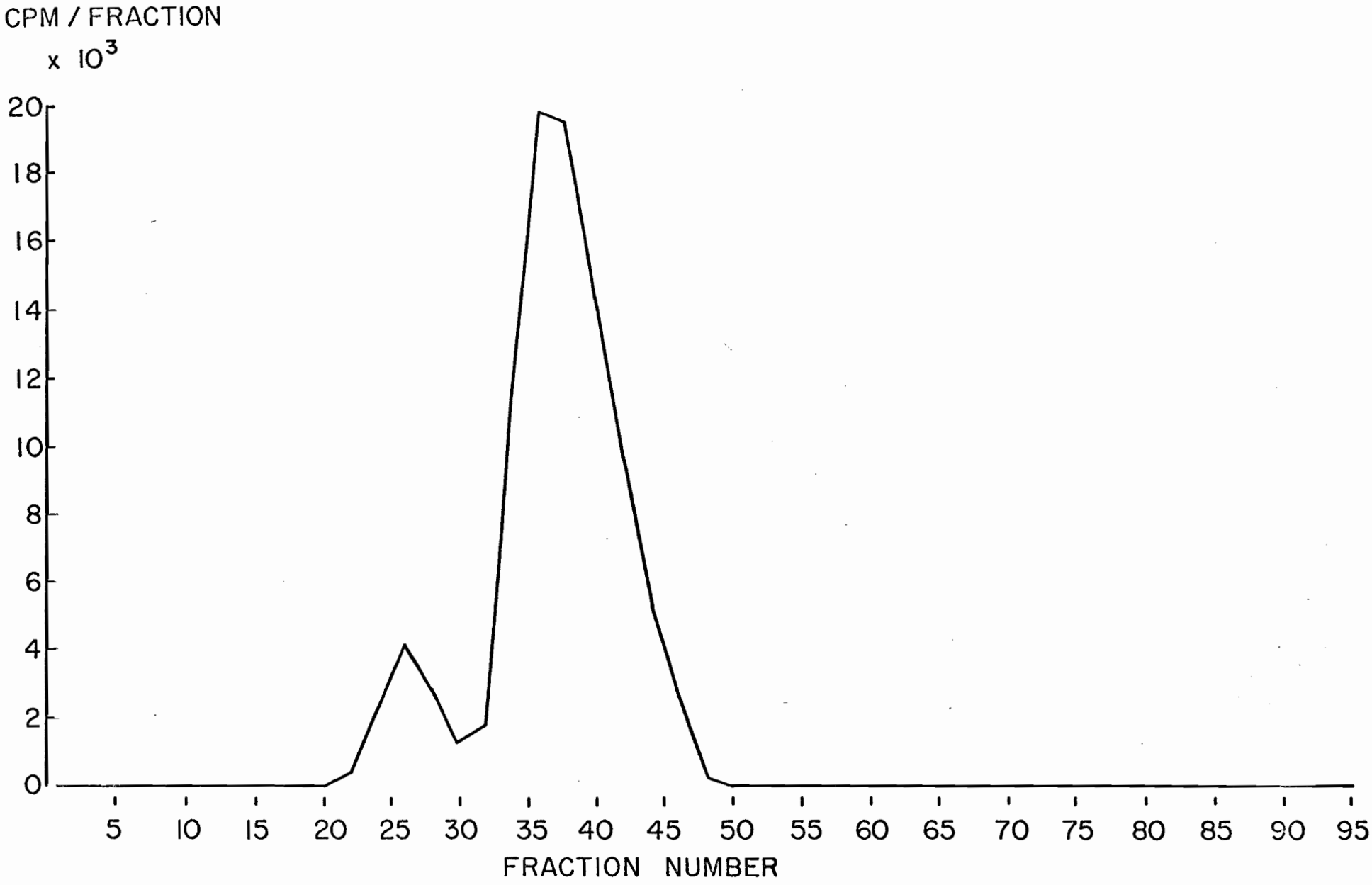

FIG. 7. CELITE COLUMN CHROMATOGRAPHY – RESIDUE OF POOL VII, EXPERIMENT 2.
SYSTEM: BENZENE: CYCLOHEXANE: METHANOL: WATER. 1:2:3:3

This residue was chromatographed on a 5 g silica gel column, using the solvent series: benzene, ether and ethyl acetate. The column was started with benzene:ether::1:1. Elution with 60, 70 and 90% ethyl acetate in ether gave 33.2 mg of a colorless glassy compound, containing 234,700 cpm. Crystallization from benzene:Skellysolve B with a trace of methanol yielded 26.1 mg of coarse needles. An infrared spectrum (KBr) was identical with that of 5β-pregnan-3α,16α-20α-triol. Recrystallization from ether:Skellysolve B with a trace of methanol gave 17.7 mg of coarse needles, m.p. 225-226°C, m.m.p. 225-226°C, standard m.p. 227-228°C. The specific activity of the compound was 10.7×10^3 cpm per mg.

EXPERIMENT 3: METABOLISM OF 7α-^{3}H-16α-HYDROXY-PROGESTERONE IN PREGNANCY

A normal 23 year old subject in the 34th week of pregnancy was given an intravenous injection of 7α-^{3}H-16α-hydroxy-progesterone containing 4.59 x 10^{6} cpm at single label settings, and 4.04 x 10^{6} cpm at double label settings. Urine was collected for 4 days. Steroid conjugates were hydrolyzed with Glusulase, 0.5 ml per 100 ml of urine. The urines were incubated for 5 days at 37°C, and a neutral ethyl acetate extract obtained. Aliquots were taken from urines and neutral extracts for counting (Table XI).

TABLE XI

EXPERIMENT 3. Injection of 7α-^{3}H-16α-hydroxy progesterone, 4.59 x 10^{6} cpm.

Radioactivity recovered - cpm x 10^{6}

	Urine	Neutral extract
Day I	2.16	1.80
II	0.53	0.33
III	0.30	0.13
IV	0.16	0.08
Total	3.15	2.34
% Dose	61%	48%

The pooled neutral extract weighed 1.63 g and contained 2.34 x 10^{6} cpm. It was chromatographed on a 225 g silica gel column, starting with 1% ethanol in methylene

chloride and continuing with increasing concentrations of ethanol. The solvent was collected in 10 ml fractions at a rate of 30 to 40 mls per hour. Fractions were pooled in groups of 4 and aliquots taken for measurement of radioactivity. Five major peaks of radioactivity were found (Fig. 8), and fractions pooled accordingly. The origin, weight and radioactivity of each residue is shown in Table XII.

The residue of pool II was chromatographed on a 30 g celite column in system A. The sample was packed on the column, using 2.5 ml of stationary phase and 5 g of celite. Mobile phase was collected in 6 ml fractions at a rate of 12 mls per hour. Sampling and counting of every fourth tube revealed 2 peaks of radioactivity (Fig. 9). The labelled compound(s) in the minor peak have not been identified. Pooling of tubes 16-25 yielded a yellow oily residue, weighing 14.4 mg and containing 72,300 cpm. An infrared spectrum (CS_2) matched that of authentic 3α-hydroxy-5β-pregnan-20-one. The residue was chromatographed on a 2 g alumina column, using the solvents n-hexane, methylene chloride and ethanol. The column was started with n-hexane: methylene chloride::1:1. Elution with n-hexane:methylene chloride::5:5, 4:6 and 3:7 gave 6.1 mg of 3α-hydroxy-5β-pregnan-20-one. The compound did not contain radioactivity. Elution of the column with n-hexane:methylene chloride::3:7, methylene chloride and 2% ethanol in methylene chloride

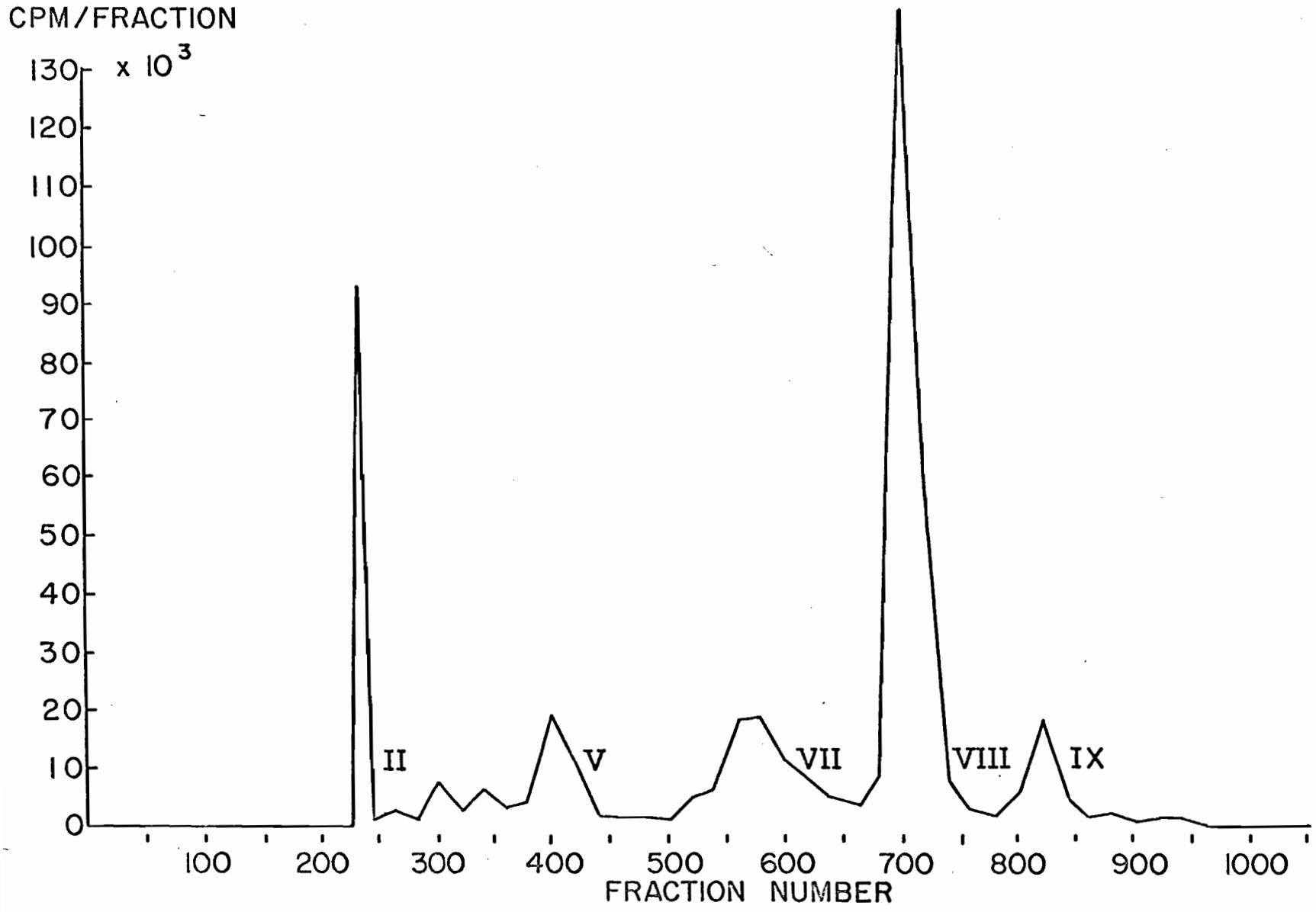

FIG.8. SILICA GEL COLUMN CHROMATOGRAPHY OF NEUTRAL EXTRACT. EXPERIMENT 3.

TABLE XII

EXPERIMENT 3. Residues from initial silica gel column.

% Ethanol	Fraction no.	Pool	
1%	1	Discarded	
	5		
	109	I	36.8 mg, 1,100 cpm
2%	210		
	228		
	240	II	66.1 mg, 89,500 cpm
	324	III	148 mg, 45,600 cpm
3%	368	IV	210 mg, 33,400 cpm
	384		
	404	V	38.4 mg, 151,500 cpm
3.5%	432		
	444		
	508	VI	44.1 mg, 20,800 cpm
4%	626	VII	97.4 mg, 268,900 cpm
	676		
6%	748	VIII	119 mg, 807,300 cpm
	800	IX	97.4 mg, 24,200 cpm
8%	847	X	97.9 mg, 117,400 cpm
	852		
10%	939	XI	263 mg, 58,000 cpm
15%	1076		

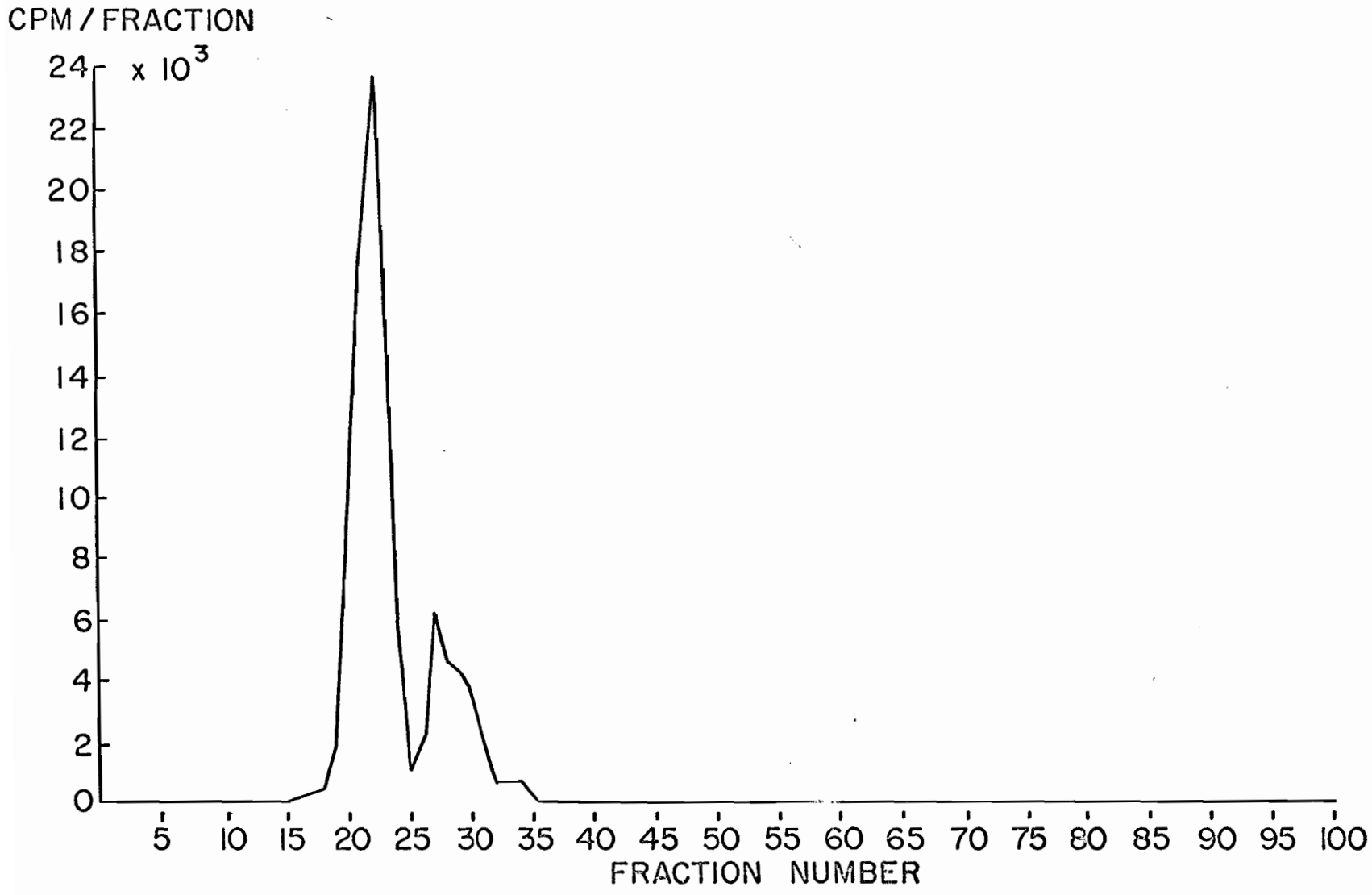

FIG. 9. EXPERIMENT 3. CELITE COLUMN CHROMATOGRAPHY. RESIDUE OF POOL II.
n-HEXANE: METHANOL: WATER. 100:90:10.

yielded 1.9 mg of yellow oil, containing 63,000 cpm. At double label settings, the count was 54,000 cpm. An infrared spectrum was identical with that of standard isopregnanolone.

A portion of the isopregnanolone, weighing 430 ug and having 12,200 cpm was acetylated with 1-^{14}C-acetic anhydride, Soln. #3. Solvents were removed under nitrogen and the residue mixed with 20.1 mg of isopregnanolone acetate. The mixture was chromatographed on a 5 g alumina column, starting with Skellysolve B. Elution with Skellysolve B:benzene::80:20, 75:25 and 65:35 gave 17.0 mg of colorless oil, containing 6400 cpm of tritium and 700 cpm of carbon-14. The compound was crystallized from acetone: n-heptane, ether:n-heptane and acetone:n-heptane. The specific activities and $^{3}H/^{14}C$ ratios of the third crystals and mother liquors (Table XIII) were constant. The predicted

TABLE XIII

EXPERIMENT 3. Crystallization of isopregnanolone acetate.

		cpm ^{3}H/mg	$^{3}H/^{14}C$			cpm ^{3}H/mg	$^{3}H/^{14}C$
Xl	1	350	14.	Ml	1	338	4.0
	2	315	13.		2	310	11.
	3	312	13.		3	314	13.

Predicted specific activity - 375 cpm ^{3}H/mg.

specific activity with respect to tritium was 596 cpm per mg after addition of carrier, and 375 cpm after chromatography

on alumina. The calculated specific activity of the urinary isopregnanolone was 5.3×10^4 cpm per mg.

The residue of pool V from the original silica gel column was chromatographed on a 70 g celite column in system B. The sample was dissolved in 15 ml of mobile phase and 1.0 ml of stationary phase and applied to the column. Mobile phase was collected in 10 ml fractions at a rate of 40 mls per hr. Aliquots were taken from every other fraction for measurement of radioactivity. Two peaks of radioactivity were found (Fig. 10), the first of which has not been identified. The residue from fractions 53 to 69, representing the second peak, weighed 4.5 mg and contained 103,700 cpm. It was chromatographed on a 2 g silica gel column, starting with benzene:ethyl acetate::80:20. Elution with benzene: ethyl acetate::40:60, 30:70 and 20:80 gave 3.7 mg of yellow oil that could not be crystallized. It was then chromatographed on a 1 g alumina column, starting with benzene. Elution with 2% ethanol in benzene gave 2.3 mg of faintly yellow oil, containing 68,000 cpm. Crystallization from acetone:n-hexane and acetone:n-heptane gave 0.54 mgms of small plates, m.p. 218-221°C, m.m.p. 218-221°C, standard m.p. 218-221°C. An infrared spectrum (KBr) was identical with that of standard 16α-hydroxy-progesterone.

The steroid used for infrared spectroscopy was recovered by dissolving the KBr disc in water and extracting

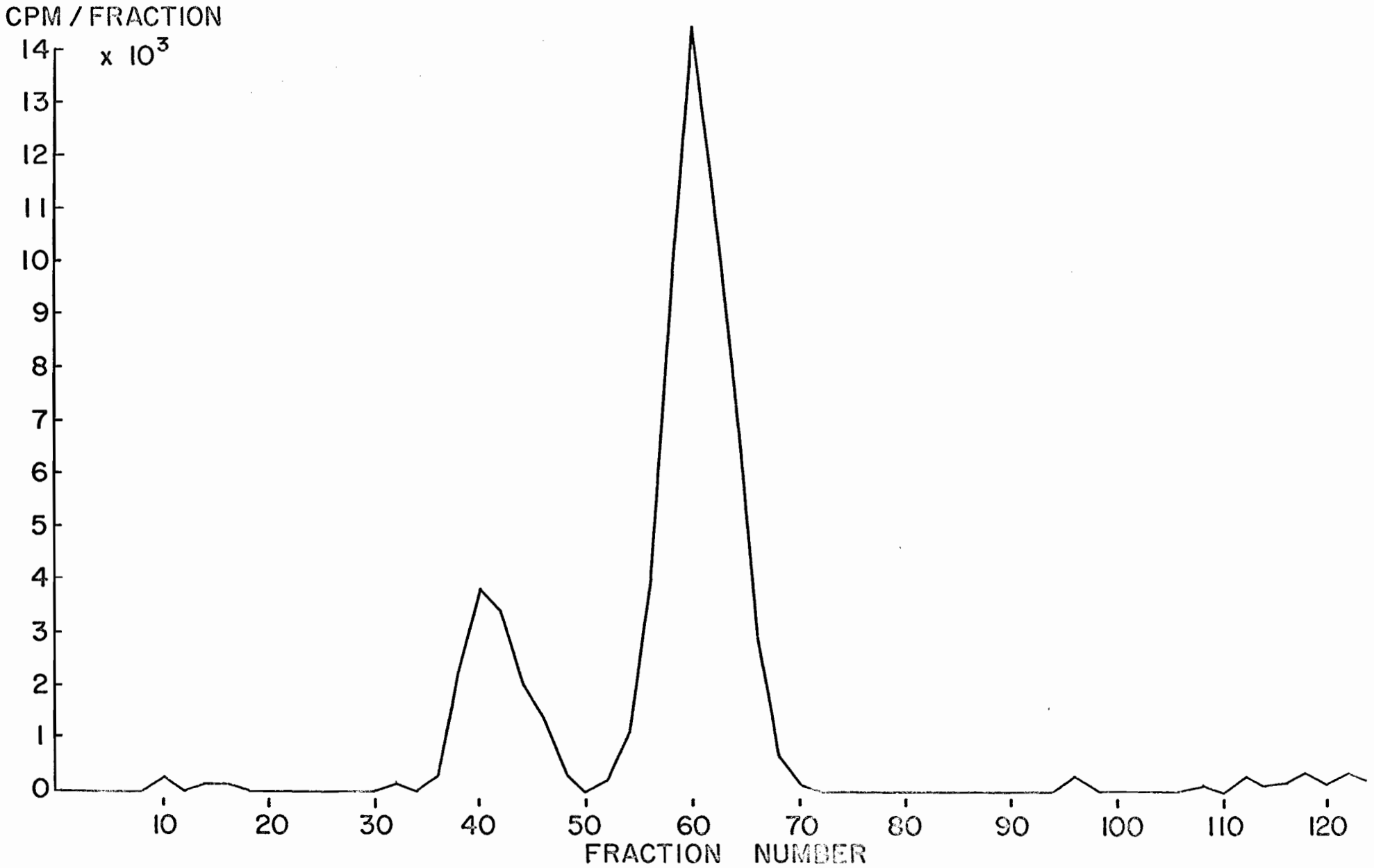

FIG. 10. EXPERIMENT 3. CELITE COLUMN CHROMATOGRAPHY. RESIDUE OF POOL V. n-HEPTANE: ETHYL ACETATE: METHANOL: WATER. 50:50:65:35.

with ethyl acetate. The residue from the disc was combined with the remaining crystals and mother liquors to give a fraction weighing 2.9 mg and containing 42,600 cpm, at double label settings. The compound was then acetylated with 1-^{14}C-acetic anhydride, Solution 2. The product was mixed with 100 mg of 16α-hydroxy-progesterone acetate and chromatographed on a 40 g silica gel column, starting with Skellysolve B:benzene::1:4. Elution with benzene:ether:: 65:35, 63:37, 60:40 and 50:50 gave 80.6 mg of clear oil. The acetate was crystallized from ether and ether:Skellysolve B. The specific activities and the value of $^{3}H/^{14}C$ (Table XIV) were constant by the second crystallization. The

TABLE XIV

EXPERIMENT 3. Crystallization of derivatives of 16α-hydroxy-progesterone.

16α-hydroxy-progesterone acetate

		cpm ^{3}H/mg	$^{3}H/^{14}C$			cpm ^{3}H/mg	$^{3}H/^{14}C$
Xl	1	426	1.7	Ml	1	444	1.2
	2	420	1.7		2	422	1.7
Predicted		439					

16α,20β-dihydroxy-Δ^{4}-pregnen-3-one, 16-acetate

		cpm ^{3}H/mg	$^{3}H/^{14}C$			cpm ^{3}H/mg	$^{3}H/^{14}C$
Xl	1	412	1.7	Ml	1	388	1.8
	2	412	1.7		2	393	1.7
Predicted		420					

predicted specific activity was calculated after the addition of carrier acetate.

A portion of the final crystals weighing 37.9 mg was dissolved in 8.0 mls of methanol and reduced with 6.1 mg of $NaBH_4$. The reduction products were dissolved in 2.0 mls of dioxane and oxidized with 28.2 mg of dichloro-dicyano-benzoquinone. The final products were chromatographed on a 5 g silica gel column, starting with benzene. Elution with benzene:ether::60:40 and 20:80, ether and ether:ethyl acetate::90:10 gave 29.4 mg of colorless oil. The compound was crystallized twice from methanol:ether:Skellysolve B. The specific activities and the value of $^{3}H/^{14}C$ (Table XIV) of the second crystals and mother liquors were constant. The predicted specific activity was based on that of the final crystals of the acetate. The calculated specific activity of the urinary 16α-hydroxy-progesterone was 6.2 x 10^4 cpm per mg. The latter figure was calculated from the value of $^{3}H/^{14}C$ of the second derivative and the specific activity of the acetic anhydride, using the expression described on page 38. It is not possible to calculate the total 16α-hydroxy-progesterone in the subjects urine.

The residue of pool VII was chromatographed on an 80 g celite column in system C. The sample was dissolved in 20 ml of mobile phase and 1 ml of stationary phase and applied to the column. Mobile phase was collected in 10 ml

fractions at a rate of 30 to 40 mls per hr. Aliquots were taken from every other tube for counting. A single peak of radioactivity was found (Fig. 11) and fractions were pooled accordingly. The residue weighed 41.7 mg and contained 270,000 cpm. It was chromatographed for 25 hrs. on Whatman #1 paper, in the system toluene:propylene glycol. Equal portions of the extract were applied to each of 6 papers. Two peaks of radioactivity were found in each paper, at average distances of 18 and 30 cms. Elution of the more polar material, VIIA, yielded 8.2 mg of residue, containing 52,500 cpm. The less polar residue, VIIB, weighed 24.3 mg and contained 134,700 cpm. Fraction VIIA was chromatographed on a 1 g silica gel column, starting with benzene. Elution with 6 and 7% ethanol yielded 2.3 mg of yellow oil, containing 31,000 cpm. An infrared spectrum (KBr) was of poor quality, and we have not been able to crystallize the material.

Fraction VIIB was chromatographed on a 2 g silica gel column, starting with benzene. Elution with 4 and 6% ethanol in benzene gave 17.2 mg of colorless oil containing 127,000 cpm. An infrared spectrum (KBr) was identical with that of 3α,16α-dihydroxy-5α-pregnan-20-one. The compound was crystallized with some difficulty from acetone:Skellysolve B, giving 6.0 mg of very fine needles. Attempts at recrystallization failed to give satisfactory crystals. Accordingly, the residue of the first mother liquors,

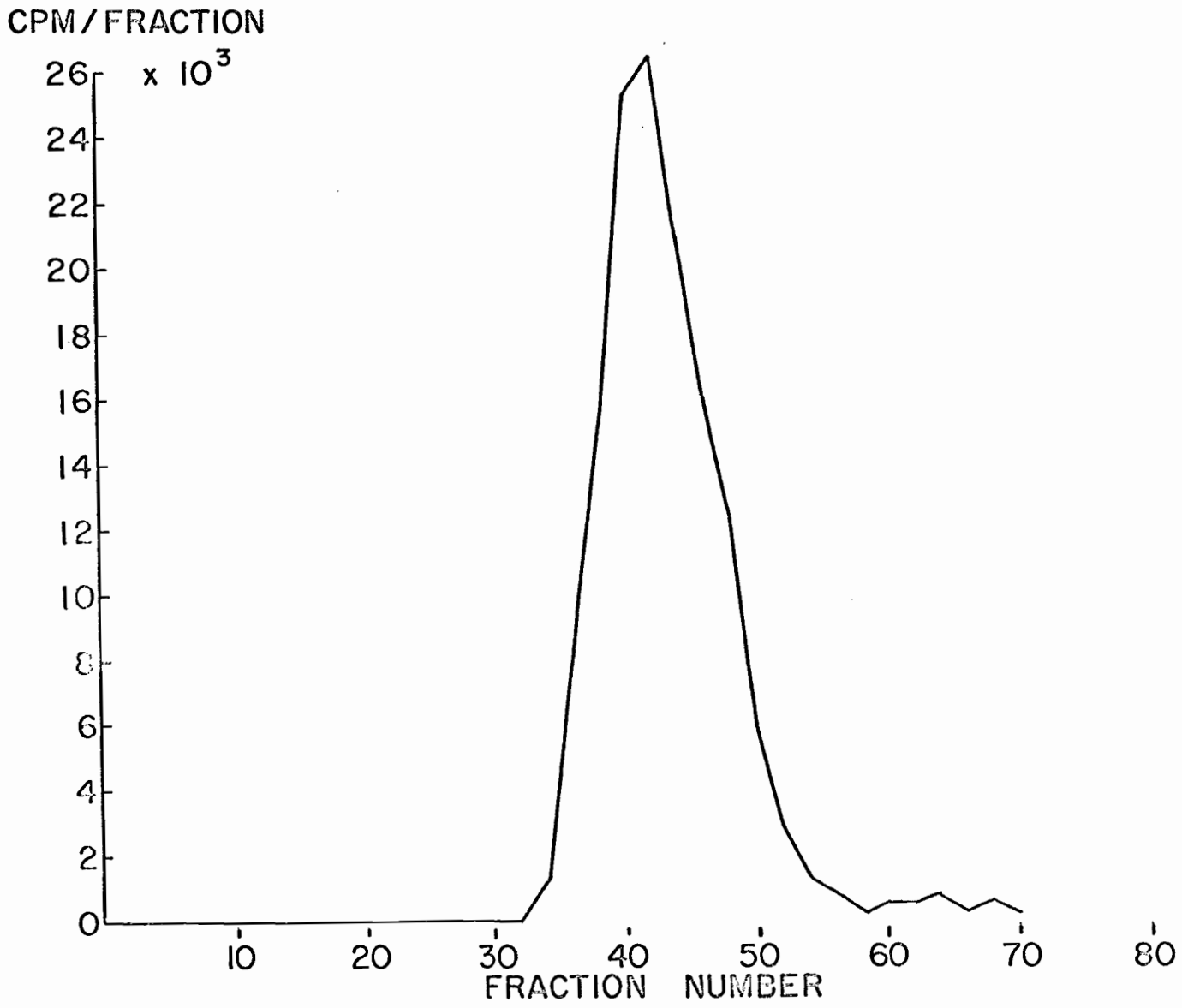

FIG. II. EXPERIMENT 3. CELITE COLUMN CHROMATOGRAPHY. RESIDUE OF POOL VII. n-HEXANE : ETHYL ACETATE : METHANOL : WATER. 50:50:65:35.

weighing 11.0 mg, was crystallized twice from acetone: Skellysolve B, yielding 1.52 mg of small plates, m.p. 226-228°C, m.m.p. 224-227°C, standard m.p. 209-211°C, s.a. 5.6×10^3 cpm per mg. An infrared spectrum (KBr) was identical with that of 3α,16α-dihydroxy-5α-pregnan-20-one.

The residue of pool VIII was chromatographed on a 100 g celite column, in system D. The sample was packed on the column, using 10 ml of stationary phase and 10 g of celite. Mobile phase was collected in 8 ml fractions at a rate of 32 mls per hr. Aliquots were taken from every other tube for counting. A single peak of radioactivity was found (Fig. 12) and fractions were pooled accordingly. The residue weighed 39.9 mg and contained 927,700 cpm. It was chromatographed for 42 hrs. on 8 strips of Whatman #1 paper, in the system toluene:propylene glycol. A single peak of radioactivity was found on each paper, at an average distance of 25 cms. Elution yielded 17.5 mg of yellow oil, containing 611,000 cpm. The residue was chromatographed on a 2 g silica gel column, starting with benzene. Elution with 6 and 7% ethanol in benzene gave 11.8 mg of colorless oil. An infrared spectrum (KBr) was identical with that of 3α,16α-dihydroxy-5β-pregnan-20-one isolated in Experiment 1. Crystallization from acetone:Skellysolve B and methanol: acetone:Skellysolve B gave 2.4 mg of fine needles, m.p. 210-211°C, m.m.p. 210-212°C, standard m.p. 210-212°C (fraction

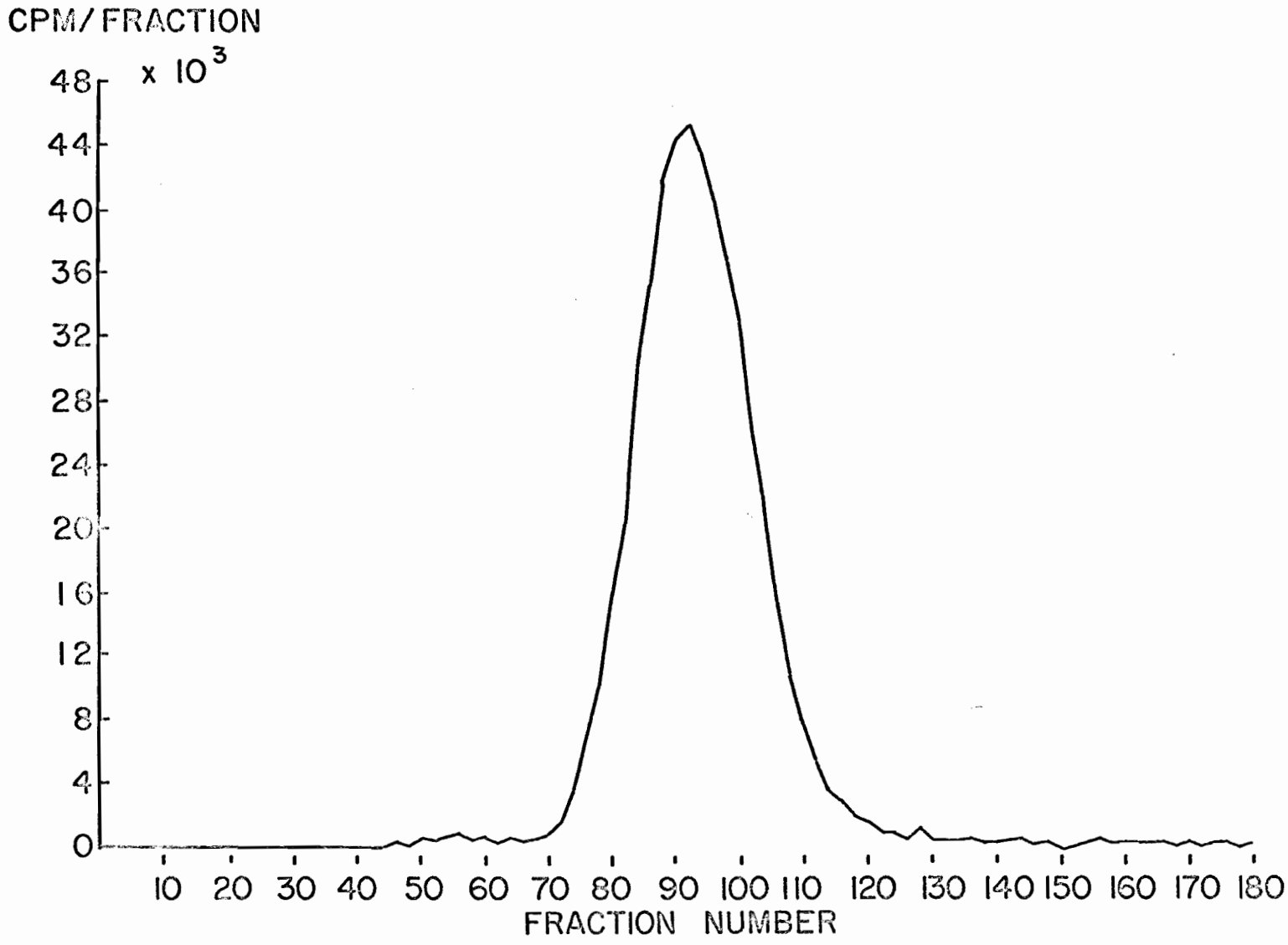

FIG.12. EXPERIMENT 3. CELITE COLUMN CHROMATOGRAPHY. RESIDUE OF POOL VIII. BENZENE : CYCLOHEXANE : METHANOL : WATER. 1:2:3:3.

B6, Expt. 1), s.a. 6.8 x 10^4 cpm per mg.

The residue of pool X was chromatographed on a 120 g celite column, in system D. The sample was packed on the column, using 10 ml of stationary phase and 10 g of celite. Mobile phase was collected in 11 ml fractions at a rate of 33 mls per hour. Aliquots were taken from every 4th tube for measurement of radioactivity. A single peak of radioactivity was found (Fig. 13) and fractions were pooled accordingly. The residue weighed 5.7 mg and contained 111,000 cpm. This residue was chromatographed for 48 hours on a 4 strips of Whatman #1 paper, in the system toluene:propylene glycol. A single peak of radioactivity was found on each paper, at distances varying from 18 to 40 cms. The areas containing radioactivity were eluted, yielding 5.7 mg of faintly yellow oil containing 75,300 cpm. The residue was chromatographed on a 1 g silica gel column, starting with benzene. Elution with 6, 8 and 10% ethanol in benzene gave 2.0 mg of colorless oil. An infrared spectrum (KBr) was identical with that of 5β-pregnan-3α,16α,20α-triol. Crystallization from acetone: Skellysolve B gave 0.71 mg of fine needles, m.p. 225-226°C, m.m.p. 224-226°C, standard m.p. 227-228°C, s.a. 4.2 x 10^4 cpm per mg.

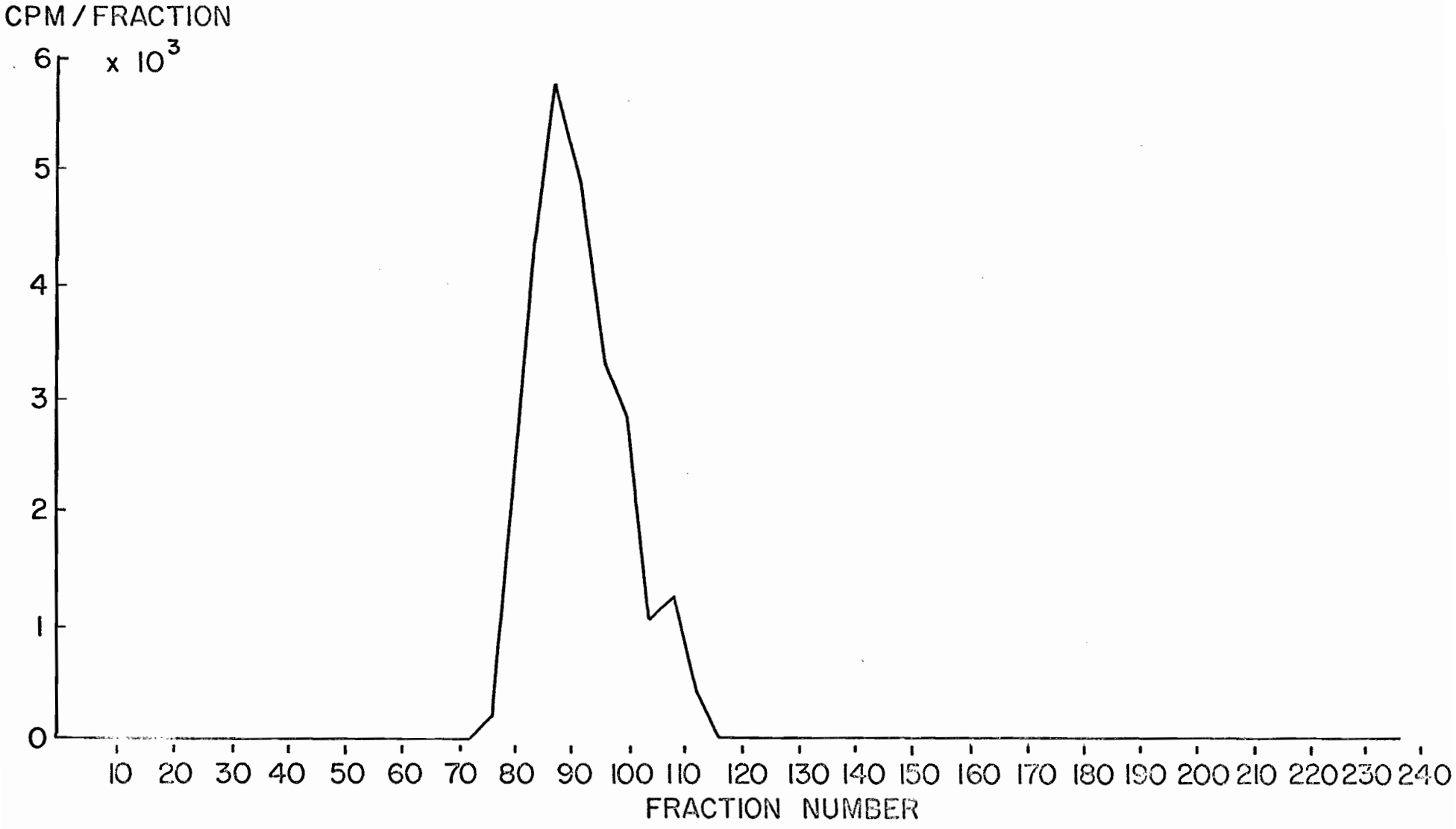

FIG. 13. EXPERIMENT 3. CELITE COLUMN CHROMATOGRAPHY. RESIDUE OF POOL X. BENZENE: CYCLOHEXANE: METHANOL: WATER. 1:2:3:3.

EXPERIMENT 4: URINARY EXCRETION OF 16α-HYDROXY-PROGESTERONE BY THE NORMAL MALE

A 38 year old normal male subject was given an intravenous injection of 7α-^{3}H-16α-hydroxy-progesterone containing 4.59 x 10^{6} cpm, by single label counting, and 4.04 x 10^{6} cpm at double label settings. Urine was collected for 4 days.

The urines were extracted with ethyl acetate prior to hydrolysis of steroid conjugates. The neutral extract so obtained contained unconjugated urinary steroids. Steroid sulphates were cleaved by the method of Burstein and Lieberman (49). Immediately following the extraction of free steroids, the residual aqueous phase and the alkali and water washes were pooled and adjusted to pH 1 with 50% sulphuric acid. After addition of sodium chloride, 20 g per cent, the aqueous was extracted with 1 vol. of freshly distilled tetrahydrofuran. The tetrahydrofuran was filtered, treated with 0.11 mls per cent of 60% perchloric acid and left in the dark at 37°C for 18 hrs. The solution was then neutralized with 0.5 mls percent of concentrated ammonium hydroxide. After evaporation of the tetrahydrofuran in vacuo, the residual water was diluted to 1000 ml with water and a neutral ethyl acetate extract obtained. This fraction contained steroids excreted as sulphates.

The residual aqueous and alkali and water washes

were pooled, adjusted to pH 4.7 and treated with 15 mg percent of a powdered β-glucuronidase preparation (Baylove Chemical). After incubation for 5 days at 37°C, a neutral ethyl acetate extract was obtained. This fraction contained steroids excreted as glucosiduronidates. Table XV shows the radioactivity recovered in urines and extracts.

TABLE XV

EXPERIMENT 4. Injection of 7α-^{3}H-16α-hydroxy-progesterone, 4.59×10^{6} cpm.

Radioactivity recovered - cpm.

		Neutral extracts		
	Urine	Free	Sulphates	Glucosiduronidates
Day I	1.47×10^{6}	6.0×10^{3}	6.8×10^{4}	1.02×10^{6}
II	0.32	1.0	1.0	0.24
III		1.0	0.9	0.13
IV	0.11	1.0	1.1	0.06
Total		9.0×10^{3}	9.8×10^{4}	1.45×10^{6}
% Dose		< 1.0%	2%	31%

The sulphate fraction weighed 1.57 g and contained 9.8×10^{4} cpm. It was chromatographed on a 225 g silica gel column, starting with 1% ethanol in methylene chloride and continuing with increasing concentrations of ethanol. The effluent was collected in 10 ml fractions at a rate of 40 mls per hr. Aliquots were taken from every fourth tube for counting. A single peak of radioactivity was found (Fig. 14).

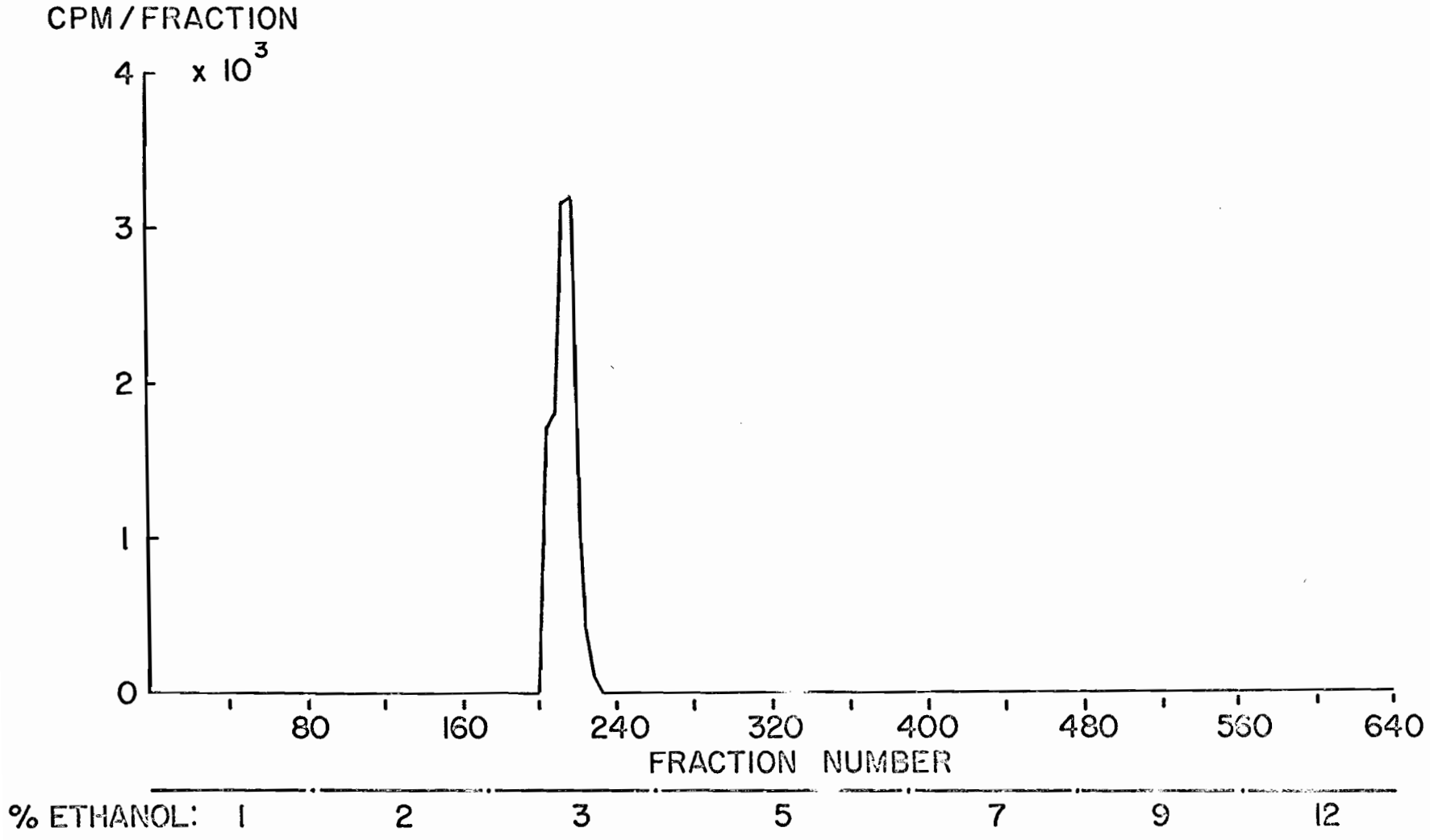

FIG. 14, EXPERIMENT 4. SILICA GEL COLUMN CHROMATOGRAPHY. SULPHATE FRACTION.

The fractions containing radioactivity were pooled, yielding 117 mg. of purple oily residue containing 54,400 cpm. This residue was applied to a 30 g alumina column and eluted with n-hexane containing increasing concentrations of 1% ethanol in methylene chloride. Elution with n-hexane:ethanol-methylene chloride::8:2 and 7:3 gave 20.1 mg of yellow oil containing 13,200 cpm. Continued elution with n-hexane: ethanol-methylene chloride::7:3 gave 11.3 mg of yellow oil containing 23,900 cpm. The latter residue, the more polar of the two, was chromatographed for eight hours on Whatman #1 paper in the system Skellysolve C:propylene glycol. Four papers were used, each with standard isopregnanolone in a separate lane. A single peak of radioactivity was found opposite the standard on each paper. The material was not further purified.

The neutral extract obtained after β-glucuronidase hydrolysis weighed 430 mg and contained 1.45×10^6 cpm. It was chromatographed on a 225 g silica gel column, using increasing concentrations of ethanol in methylene chloride. The effluent was collected in 10 ml fractions at the rate of 40 mls per hour. Aliquots were taken from every fourth fraction for counting. Five major peaks were found (Fig. 15) and fractions were pooled accordingly.

The residue from tubes 296-348 (peak V) weighed 24.7 mg and contained 1.22×10^5 cpm. It was chromatographed on

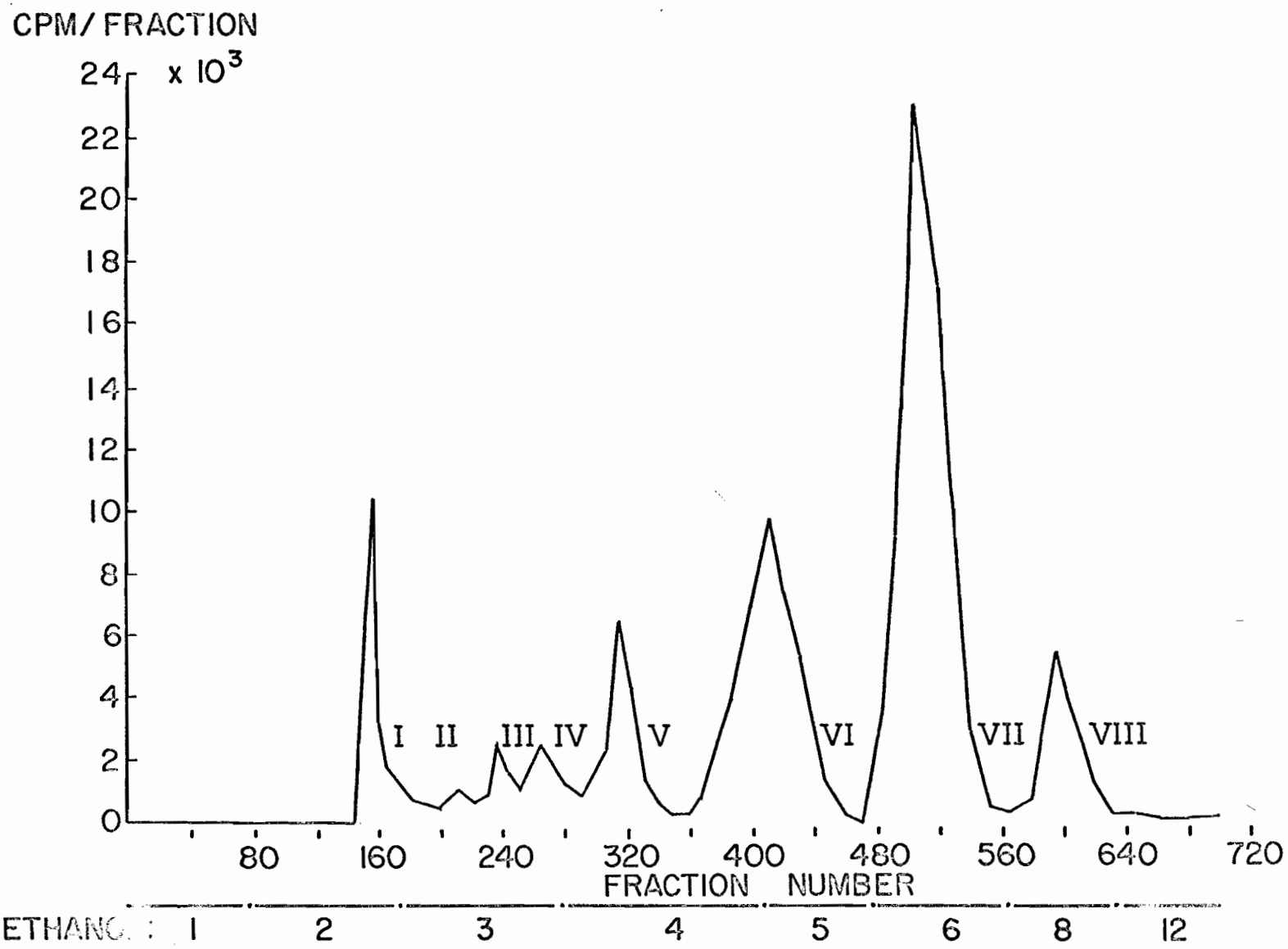

FIG. 15. EXPERIMENT 4. SILICA GEL CHROMATOGRAPHY. GLUCOSIDURONIDATE FRACTION.

a 30 g celite column, in system B. The sample was dissolved in 5 ml of mobile phase and 0.2 ml of stationary phase and applied directly to the column. Mobile phase was collected in 10 ml fractions at the rate of 30 mls per hour. Aliquots were taken from every other tube for counting. Two peaks of radioactivity were found (Fig. 16). In error, fractions 4 to 23, including both peaks, were pooled. The residue weighed 15.0 mg and contained 113,800 cpm.

The residue was chromatographed for 9 hrs. on 8 strips of Whatman #1 paper, in the system toluene:propylene glycol. A single peak of radioactivity was found on each paper, at an average distance of 17.0 cms. Elution of the areas containing radioactivity gave 5.2 mg of yellow oily residue containing 66,400 cpm. This residue was chromatographed on a 2 g alumina column, starting with benzene. Elution with 2% ethanol in benzene gave 0.87 mg of yellow oil containing 54,300 cpm. The same material counted at double label settings contained 47,800 cpm. The residue was acetylated in 0.2 ml of pyridine and 0.3 ml of 1-^{14}C-Acetic anhydride, solution 1. Solvents were removed with a stream of nitrogen and the residue mixed with 100 mg of 16α-hydroxyprogesterone acetate. The mixture was crystallized from ether:methanol, ether, ether:Skellysolve B and methanol:ether: Skellysolve B. The specific activities and the value of $^{3}H/^{14}C$ of the fourth crystals and mother liquors (Table XVI)

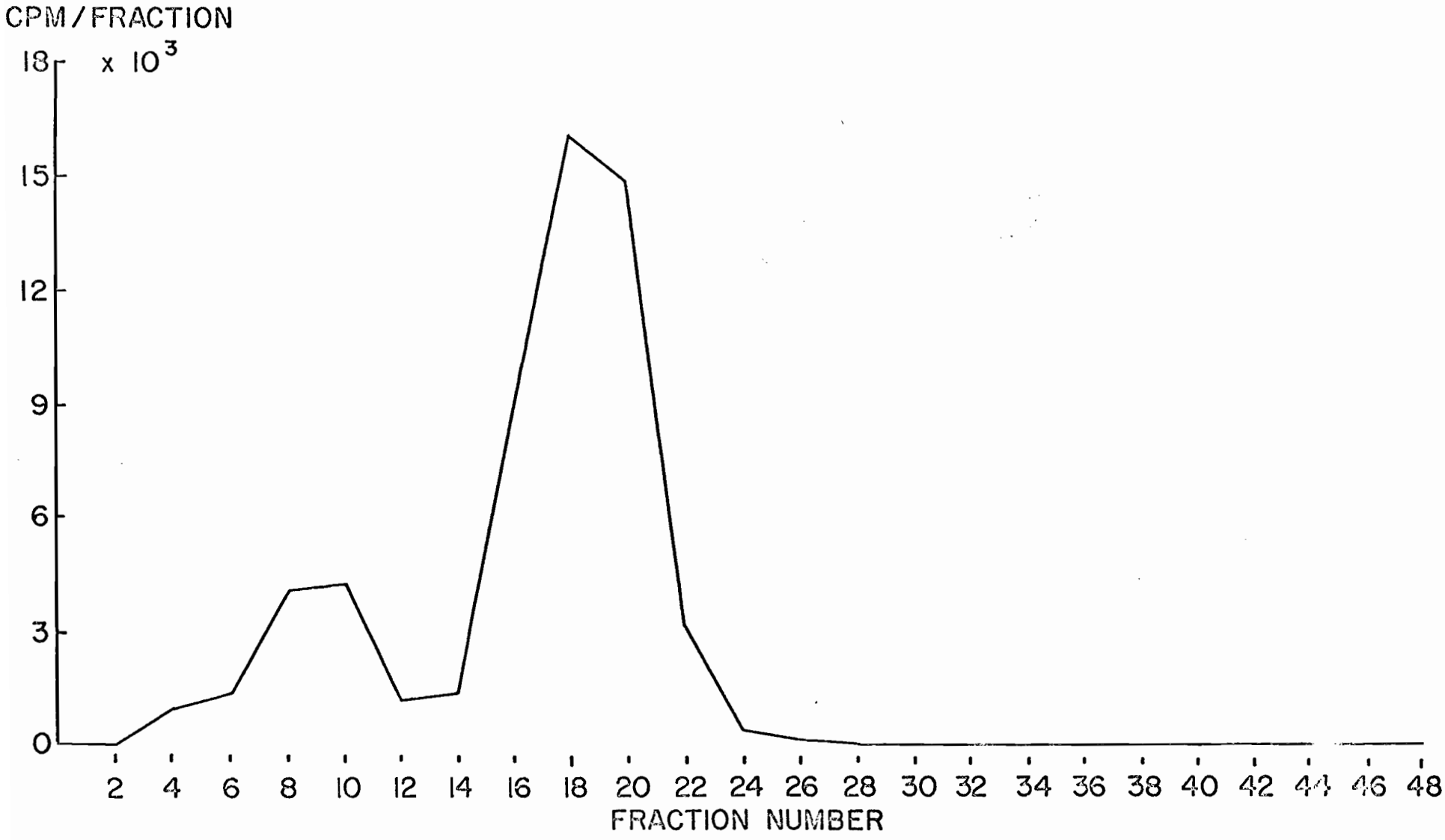

FIG. 16. EXPERIMENT 4. CELITE COLUMN CHROMATOGRAPHY. RESIDUE OF POOL V.
n-HEPTANE: ETHYL ACETATE: METHANOL: WATER. 50:50:65:35.

TABLE XVI

EXPERIMENT 4. Crystallization of 16α-hydroxy-progesterone derivatives.

16α-hydroxy-progesterone acetate

		cpm ^{3}H/mg	^{3}H/^{14}C			cpm ^{3}H/mg	^{3}H/^{14}C
Xl	1	388	2.7	Ml	1	617	.08
	2	395	3.9		2	418	.40
	3	389	4.5		3	396	2.2
	4	393	4.7		4	320	4.3

Predicted - 400 cpm ^{3}H/mg.

16α,20β-dihydroxy-Δ^4-pregnen-3-one, 16 acetate

		cpm ^{3}H/mg	^{3}H/^{14}C			cpm ^{3}H/mg	^{3}H/^{14}C
Xl	1	396	4.9	Ml	1	352	4.6
	2	384	4.6		2	374	4.6

Predicted - 391 cpm ^{3}H/mg.

were constant, within the limitations of error of measurement. The predicted specific activity was calculated after the addition of carrier acetate.

The final crystals and mother liquors were pooled and reduced with $NaBH_4$. A solution of 26.6 mg of steroid in 5.3 ml of methanol was treated with 4.3 mg of $NaBH_4$. The products were dissolved in 2.0 ml of dioxane together with 20.5 mg of dichloro-dicyano benzoquinone. The residue obtained after oxidation weighed 29.4 mg. It was chromatographed on a 5 g silica gel column, starting with benzene. Elution with benzene:ether::6:4 and 2:8, and with ether gave

23.5 mg of colorless oil. The product was crystallized twice from methanol:ether:Skellysolve B. The specific activities and the value of $^{3}H/^{14}C$ (Table XVI) of the second crystals and mother liquors were constant, within the limitations of the error of measurement. The predicted specific activity was calculated from the specific activity of the acetate. The calculated specific activity of the urinary 16α-hydroxy-progesterone was 1.66×10^{6} cpm per mg.

EXPERIMENT 5: THE ORIGIN OF 16α-HYDROXY-PROGESTERONE IN PREGNANCY.

A normal 23 year old subject in the 26th week of pregnancy was given an intravenous injection containing 7α-^{3}H-16α-hydroxy-progesterone, 2.85 x 10^{6} cpm, and 4-^{14}C-progesterone, 1.85 x 10^{6} cpm. Urine was collected for 4 days and stored in the frozen state until processed.

The urinary steroid conjugates were hydrolyzed with Glusulase, 0.5 ml per 100 ml of urine, and neutral ethyl acetate extracts obtained. Aliquots were taken from urines and extracts for measurement of radioactivity (Table XVII).

TABLE XVII

EXPERIMENT 5: Radioactivity recovered in urine and extracts

	Urine		Neutral extracts	
	cpm ^{3}H	cpm ^{14}C	cpm ^{3}H	cpm ^{14}C
Day I	1.29 x 10^{6}	1.83 x 10^{5}	4.10 x 10^{5}	1.93 x 10^{5}
II	0.13	0.45	2.41	0.43
III	0.14	0.41	1.49	0.24
IV	0.04	0.32	0.41	0.08
Total	1.60 x 10^{6}	3.01 x 10^{5}	6.41 x 10^{5}	1.68 x 10^{5}

NOTE: Tritium counts are not corrected for quenching.

Only the carbon-14 counts have been corrected for quenching. An error in the addition of internal standard prevented the correction of the tritium counts.

The combined neutral extract weighed 1.07 g and contained 6.41×10^5 cpm of tritium and 1.68×10^5 cpm of carbon-14. It was chromatographed on a 240 g silica gel column, using increasing concentrations of ethanol in methylene chloride. The effluent was collected in 10 ml fractions, at the rate of 40 mls per hour. Aliquots were taken from every fourth tube for counting. Five major peaks of tritium were found (Fig. 17) and fractions were pooled accordingly.

The residue from tubes 389-468 (peak IV) weighed 29.3 mg and contained 7.7×10^4 cpm of tritium and 5.5×10^3 cpm of carbon-14. It was chromatographed on a 30 g celite column in system B. The sample was dissolved in 10 ml of mobile phase and 0.2 ml of stationary phase and applied to the column. Mobile phase was collected in 5 ml fractions, at the rate of 15 ml per hour. Aliquots were taken from every fourth tube for counting. Two major peaks of tritium were present (Fig. 18). The residue from the fractions 32-47 weighed 5.9 mg and contained 28,100 cpm of tritium. The residue was chromatographed on 6 g of alumina, starting with benzene. Elution with 2% ethanol in benzene gave 1.2 mg of colorless oil containing 22,500 cpm of tritium. A portion of this residue was counted until 100,000 cpm had accumulated in the tritium channel, at which time there was no evidence of carbon-14 in the vial.

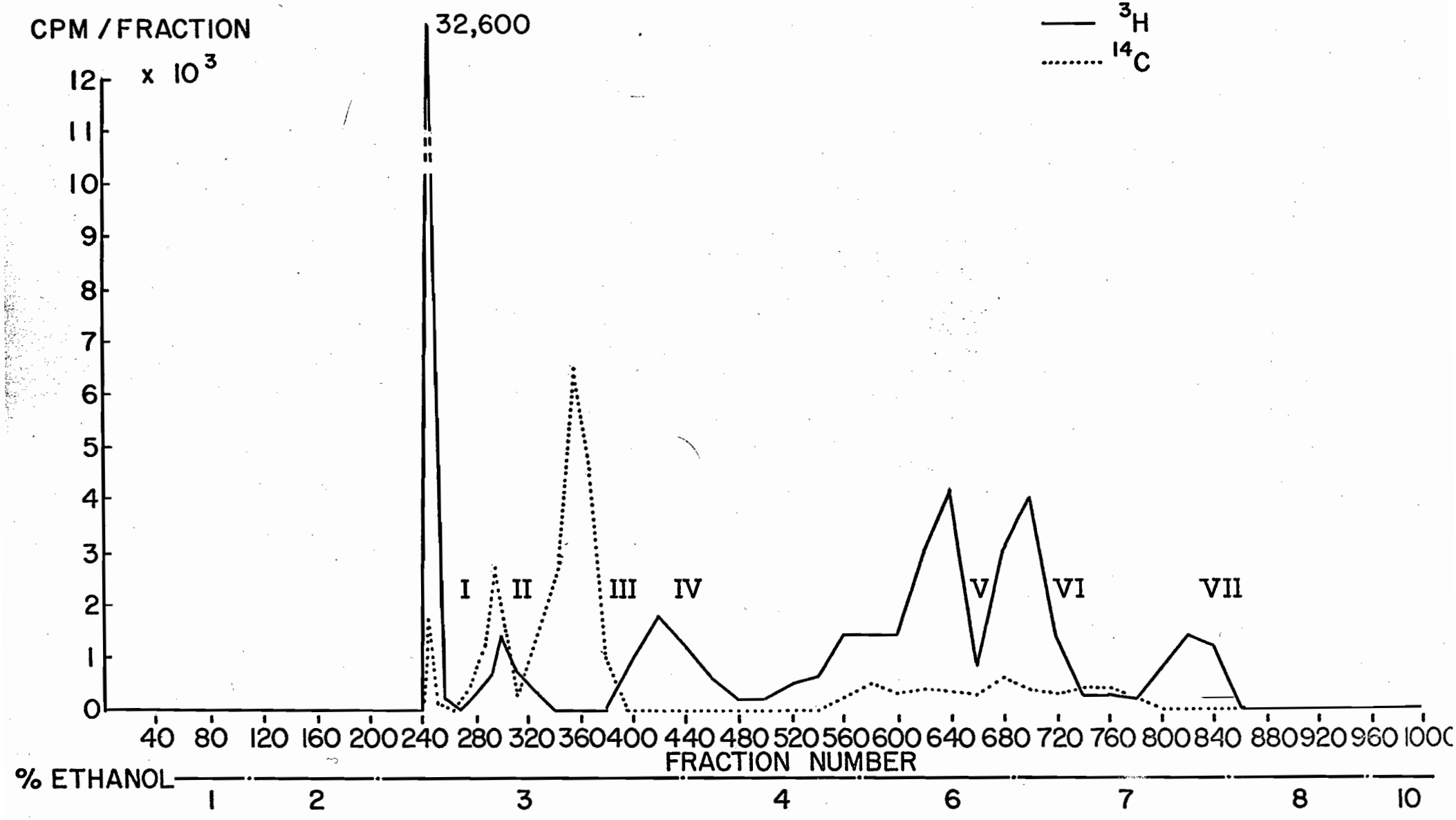

FIG. 17. EXPERIMENT 5. SILICA GEL COLUMN CHROMATOGRAPHY. NEUTRAL EXTRACT.

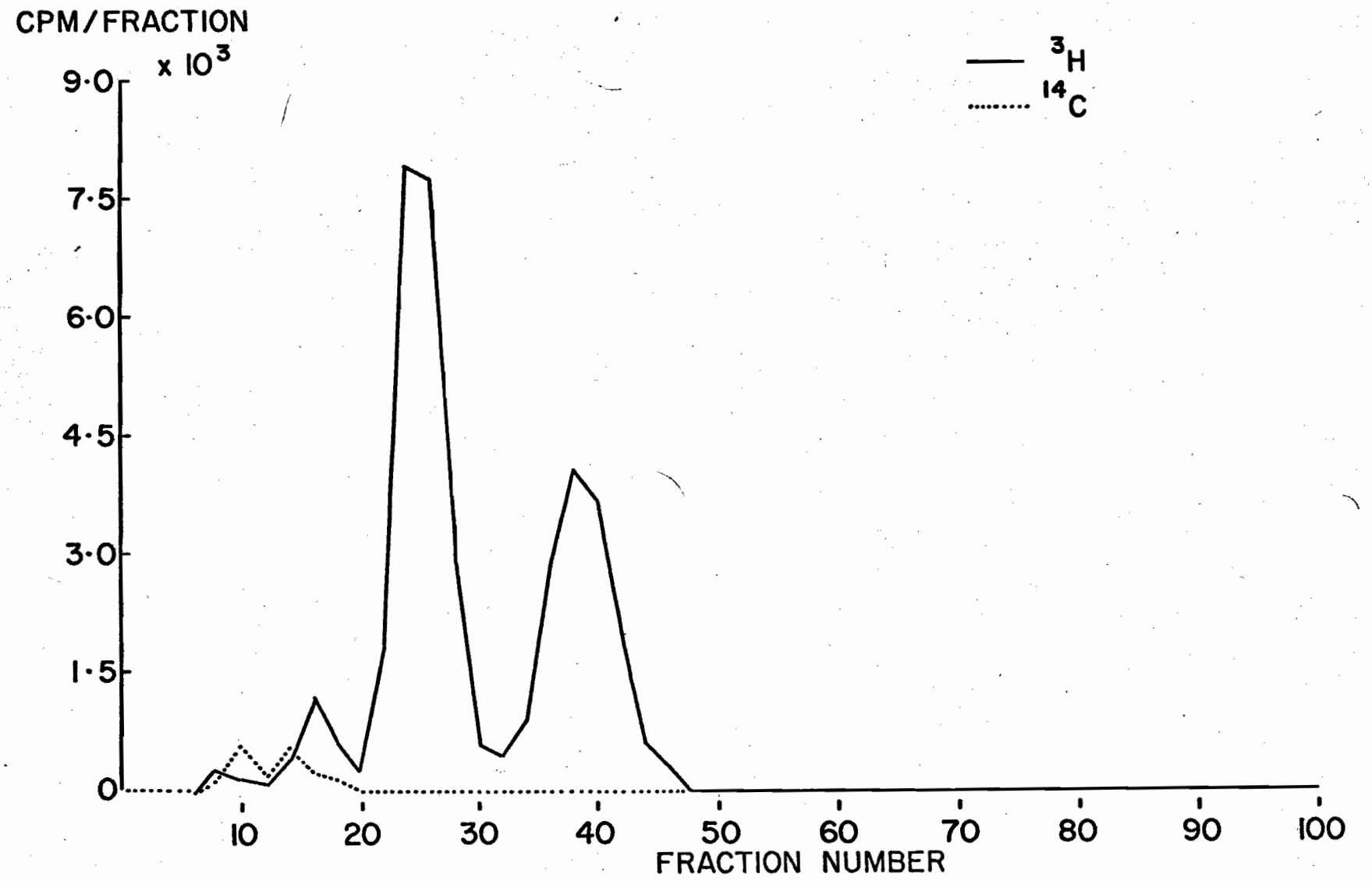

FIG. 18. EXPERIMENT 5. CELITE COLUMN CHROMATOGRAPHY. RESIDUE OF POOL IV.
n-HEPTANE : ETHYL ACETATE : METHANOL : WATER. 50:50:65:35.

The residue was then mixed with 89.0 mg of 16α-hydroxy-progesterone. The mixture was crystallized from acetone:methanol, acetone:methanol:Skellysolve B and twice from ethanol:Skellysolve B. The specific activities of the fourth crystals and mother liquors (Table XVIII) were

TABLE XVIII

EXPERIMENT 5: Crystallization of 16α-hydroxy-progesterone

Specific activity - cpm ^{3}H/mg.

		Crystals	Mother liquors
Free	1.	218	423
	2.	208	252
	3.	207	226
	4.	207	223
Predicted - 250 cpm/mg.			
Acetate	1.	189	159
	2.	179	178
	3.	175	178
Predicted - 184 cpm/mg.			

constant, within the limitations of error of measurement. The predicted specific activity was calculated after the addition of carrier.

The final crystals, 38.2 mg, were acetylated in 0.5 ml of pyridine and 0.25 ml of acetic anhydride. Extraction with ethyl acetate following addition of acidified water yielded a residue weighing 45.1 mg. It was

crystallized twice from ethanol:Skellysolve B and once from ether:ethanol. The specific activities (Table XVIII) of the second and third crystals and mother liquors were constant, within the limitations of error of measurement. The predicted specific activity was based on that of the free compound.

EXPERIMENT 6: THE ORIGIN OF 16α-HYDROXY-PROGESTERONE IN PREGNANCY

A 23 year old normal subject in the 32nd week of pregnancy was given an intravenous injection containing 7α-^{3}H-16α-hydroxy-pregnenolone, 5.31 x 10^{6} cpm, and 4-^{14}C-16α-hydroxy-progesterone (lot II), 1.08 x 10^{6} cpm. Urine was collected for nine days.

The individual urines were first extracted with tetrahydrofuran, to extract steroid conjugates and to achieve a more concentrated aqueous solution. Urine was adjusted to pH 1.0 with 50% sulphuric acid, treated with sodium chloride, 20 g per 100 ml, and extracted with an equal volume of freshly distilled tetrahydrofuran. The organic phase was distilled *in vacuo* at 40°C until no tetrahydrofuran remained. The water remaining was diluted to 400 ml with water, the steroid conjugates hydrolyzed with Glusulase, 0.5 ml per cent, and a neutral ethyl acetate extract obtained. Aliquots were taken from urines and neutral extracts for measurement of radioactivity (Table XIX).

The combined neutral extract weighed 3.91 g and contained 2.76 x 10^{6} cpm of tritium and 5.35 x 10^{5} cpm of carbon-14. It was chromatographed on a 1 kg column of silica gel, using increasing concentrations of ethanol in methylene chloride. The effluent was collected in 15 ml fractions at

TABLE XIX

EXPERIMENT 6: Radioactivity recovered in urine and neutral extracts of days 1-5.

	Urine		Neutral extracts	
	cpm ^{3}H	cpm ^{14}C	cpm ^{3}H	cpm ^{14}C
Day 1	2.36×10^{6}	4.25×10^{5}	2.01×10^{6}	3.76×10^{5}
2	0.45	1.55	0.38	1.28
3	0.22	0.87	0.22	0.78
4	0.10	0.38	0.09	0.29
5	0.05	0.28	0.06	0.24
Total	3.18×10^{6}	7.33×10^{5}	2.76×10^{6}	5.35×10^{5}

the rate of 150 mls per hr. Aliquots were taken from every fifteenth tube for counting. Five peaks of carbon-14 were found (Fig. 19), although the peak usually associated with 16α-hydroxy-progesterone was not apparent. Fractions 991 to 1300 yielded 532 mg of brown, oily residue, containing 48,800 cpm of tritium and 24,500 cpm of carbon-14. The residue was chromatographed on a 200 g celite column in system B. The sample was packed on the column, using 8 mls of stationary phase and 8 g of celite. Mobile phase was collected in 10 ml fractions at the rate of 40 mls per hour. Fractions were pooled in groups of four and aliquots taken for counting. Several peaks of radioactivity were found (Fig. 20). Fractions 120 to 164 were pooled, yielding 31.2 mg of yellow oily residue containing 19,100 cpm of tritium and 2900 cpm of carbon-14.

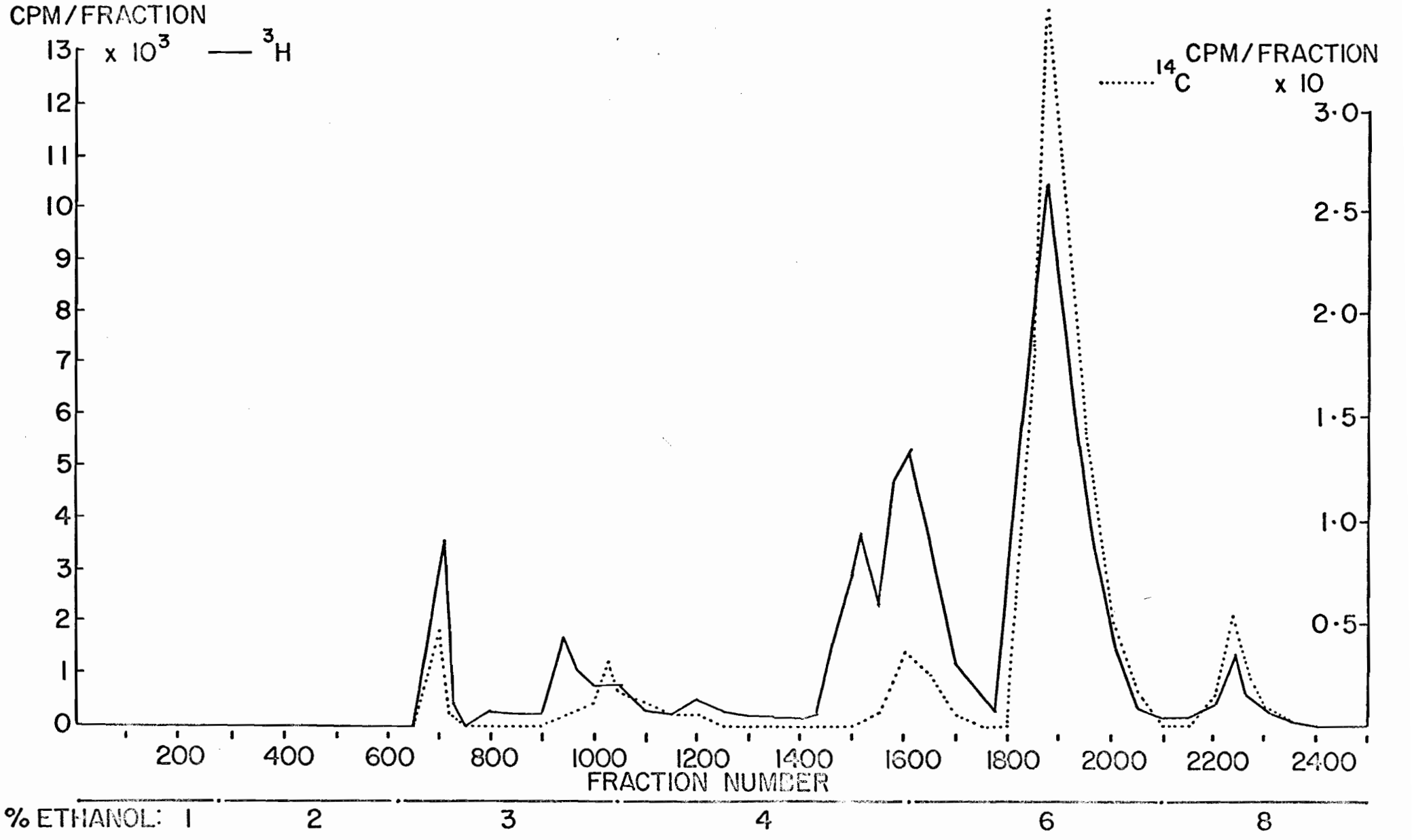

FIG.19. EXPERIMENT 6. SILICA GEL COLUMN CHROMATOGRAPHY. NEUTRAL EXTRACT.

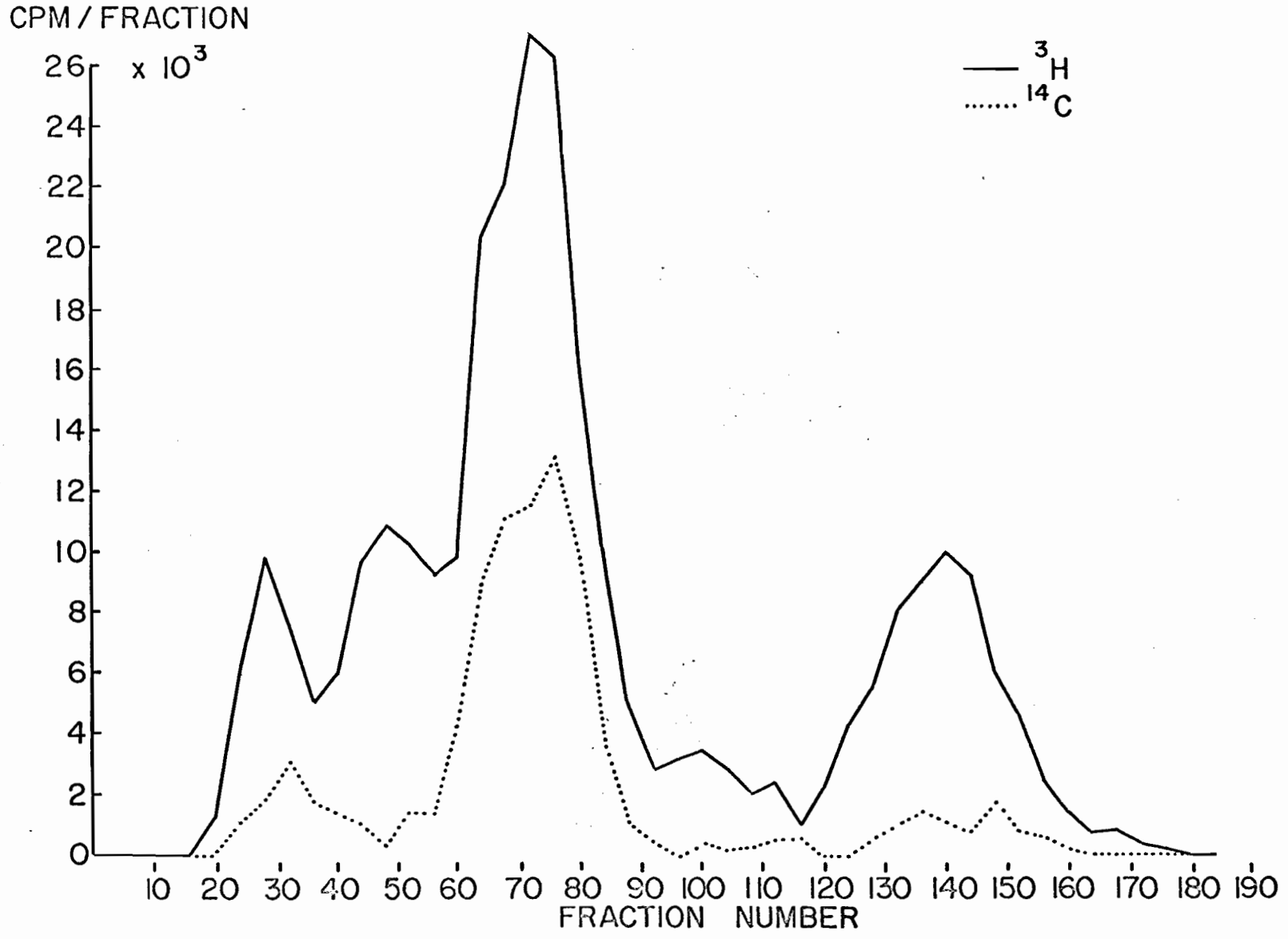

FIG.20. EXPERIMENT 6. CELITE COLUMN CHROMATOGRAPHY
n-HEPTANE : ETHYL ACETATE : METHANOL : WATER. 50:50:65:35

The residue was chromatographed for 10 hours on 5 strips of Whatman #1 paper in the system toluene:propylene glycol. A single peak of radioactivity and a corresponding U.V. absorbing area were found on each paper. Elution of the areas containing radioactivity gave 7.1 mg of yellow oil, containing 14,400 cpm of tritium and 1800 cpm of carbon-14. To this material was added 18 mg of 16 -hydroxy-progesterone. The mixture was chromatographed on a 5 g alumina column, starting with benzene. Elution with 1 and 1.5% ethanol in benzene gave 19.1 mg of colorless oil. The compound was crystallized twice from ethanol: Skellysolve B and once from acetone. The specific activities and the value of $^{3}H/^{14}C$ (Table XX) were constant by the

TABLE XX

EXPERIMENT 6: Crystallization of 16α-hydroxy-progesterone

Free

		cpm ^{3}H/mg	$^{3}H/^{14}C$			cpm ^{3}H/mg	$^{3}H/^{14}C$
Xl	1	753	6.7	Ml	1	311	6.9
	2	778	6.7		2	590	7.0
	3	770	6.6		3	793	7.1

Predicted - 665 cpm ^{3}H/mg.

Acetate

		cpm ^{3}H/mg	$^{3}H/^{14}C$			cpm ^{3}H/mg	$^{3}H/^{14}C$
Xl	1	640	6.6	Ml	1	519	7.1
	2	635	6.9		2	606	6.9

Predicted - 690 cpm ^{3}H/mg.

third crystallization. The predicted specific activity was calculated after addition of carrier. The calculation included the weight of the carrier and the weight of the isolated fraction. The final crystals and mother liquors were pooled and acetylated in 0.4 ml of pyridine and 0.2 ml of acetic anhydride. Solvents were removed under a stream of nitrogen, leaving 10 mg of residue. The acetate was crystallized from ethyl acetate:Skellysolve B and ethanol: Skellysolve B. The specific activities and the values of $^{3}H/^{14}C$ (Table XX) of the second crystals and mother liquors were constant, within the limitations of error of measurement. The predicted specific activity was based on that of the free compound. The ratio of tritium to carbon-14 in the isolated 16α-hydroxy-progesterone was higher than that of the injected compounds.

DISCUSSION

PREPARATION OF LABELLED SUBSTRATES

The ability of certain micro-organisms to introduce hydroxyl groups into the steroid molecule is well known (55) and many groups, especially in the drug industry, have taken advantage of these organisms. The use of micro-organisms in the present work provided a convenient and practical method of synthesis of labelled 16α-hydroxy compounds of relatively high specific activity. In addition to the compounds described, we were able to make 16α-hydroxy-Δ^4-androsten-3,17 dione and 3β,16α-dihydroxy-Δ^5-androsten-17-one. The 3-sulphate of 3β,16α-dihydroxy-Δ^5-androstene-17-one has been made as well (56). An attempt to make 16α-hydroxy-oestrone was unsuccessful (57). The synthesis of specifically labelled 16α-hydroxy steroids by chemical means is a formidable task. The microbiological method on the other hand has the advantages of simplicity and a high degree of specificity. With one exception, the incubations gave rise to a maximum of two products. This specificity was of significance in the subsequent purification of the desired compound. A high degree of purity was

achieved rather easily with 2 or 3 chromatographic procedures. The overall efficiency of the method varied from 8 to 70%. In no instance did crystallization of the product reveal measurable impurities.

ISOLATION OF URINARY METABOLITES

The intravenous injection of labelled steroids was, of course, the most significant step in the experiments. The label not only provides evidence for the relationship of urinary steroid to injected steroid, but also eliminates almost completely the need to isolate and identify a large number of unrelated compounds.

The technique of silica gel chromatography of the crude extracts proved to be the most useful development in the isolation procedures. A high degree of resolution of labelled metabolites was achieved by the use of large columns developed over long periods (9 to 14 days). Rather small fractions were collected, to take advantage of the resolution on the columns. The efficiency of these columns simplified subsequent purification by permitting a wider choice of solvent systems for celite column chromatography, and reducing the number of paper systems required. In certain parts of Experiment 2, the celite columns were omitted altogether, although at the expense of an increased number of paper chromatograms. Comparison of the initial

silica gel column of Experiment 3 with that of Experiment 1, which was developed and eluted in only 12 hours, illustrates the advantage of prolonging the development time. The results of Experiment 1 however provide evidence that the metabolites isolated in subsequent experiments were not artefacts resulting from prolonged exposure to silica gel.

All the reported metabolites were crystallized and identified by melting points and infrared spectra, and by mixed melting points with standards. In some instances, steroids isolated in Experiment 1 were used as standards for compounds isolated in later studies.

EXPERIMENT 1

The experiment was designed to facilitate the isolation and identification of metabolites of 16α-hydroxy-progesterone, and in this regard was quite successful. The administration of oral and intravenous doses at different times prevented the possible influence of a huge oral load of 16α-hydroxy-progesterone on the metabolism of the circulating labelled compound. However it must be noted that the weight of the intravenously administered labelled steroid, 2 mg, is undoubtedly outside the physiological range, and may have influenced somewhat the subsequent distribution of radioactivity among the isolated compounds.

Of the five labelled steroids isolated from urine, one, isopregnanolone, is a known metabolite of 16α-hydroxy-

progesterone, and is believed to be derived from an intermediate Δ^{16}-steroid. We have no evidence bearing on the identity of the immediate precursor of isopregnanolone. The remaining four metabolites, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one, 5 -pregnan-3α,16α,20α-triol and 5β-pregnan-3α,16α,20α-triol have all been reported previously, although their precursors were a matter of speculation.

The specific activities of the 5 compounds (Table XXI) show considerable variation. The variation is probably

TABLE XXI

Experiment 1

Specific activities of isolated urinary metabolites

Compound	Specific activity - $cpm^{14}C/mg.$
isopregnanolone	1.4×10^3
3α,16α-dihydroxy-5α-pregnan-20-one	1.1×10^3
5α-pregnan-3α,16α,20α-triol	1.3×10^3
3α,16α-dihydroxy-5β-pregnan-20-one	2.8×10^3
5β-pregnan-3α,16α,20α-triol	2.4×10^3

due to differences in the rates of metabolism of the oral and intravenous steroid in the various metabolic pathways. It is unlikely that the subject produced other precursors in quantities sufficient to cause such differences in specific activities.

EXPERIMENT 2

It was our object in this study to confirm the findings of Experiment 1, using somewhat improved methods, particularly the initial silica gel column. The study was not completely successful. Isopregnanolone was not found in the urine extracts, for reasons that are unknown to us. It is not possible to say whether the absence of this compound is related to the fact that the oral and intravenous doses were given at the same time. We were not able to detect the presence of 5α-pregnan-3α,16α,20α-triol. The three remaining metabolites of Experiment 1 were identified satisfactorily.

This experiment did, however, bring to our attention the possibility that 16α-hydroxy-progesterone might be found in urine. The isolation of 16α-hydroxy-progesterone, even after an oral load, suggested that its metabolism might be delayed sufficiently to permit excretion in measurable amounts in more physiological states. The effects of the 16 -hydroxy group on metabolism of steroids have been mentioned (17).

EXPERIMENT 3

It is well known that the normal pregnant subject excretes large amounts of oestriol. In recent years it has

been shown that a significant portion of the oestriol of pregnancy is derived from neutral 16α-hydroxylated precursors (23, 58, 59). The ability of certain foetal tissues to hydroxylate steroids in the 16α-position has been discussed. Furthermore, two of the known urinary neutral 16α-hydroxy steroids were first isolated from pregnancy urine. These observations suggested that studies of the metabolites of 16α-hydroxy-progesterone in pregnancy would be fruitful. Four such metabolites were isolated and identified: isopregnanolone, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one and 5β-pregnan-3α,16α, 20α-triol. Of the five compounds isolated in Experiment 1, only 5α-pregnan-3α,16α,20α-triol was not found in this study.

The isolation of 16α-hydroxy-progesterone from urine is of some interest. Urinary steroids retaining the Δ^4-3 ketone are not common. Most of these steroids, such as cortisol (60) and aldosterone (61) are present in small quantities. The recovery of 16α-hydroxy-progesterone in this experiment was certainly not quantitative. However the isolation of 0.5 mg of the compound suggests that it was excreted in amounts that exceed those reported for other Δ^4-3 keto-steroids, with the exception of 6β-hydroxy-cortisol (62).

The specific activities of the isolated compounds are shown in Table XXII. The figures for isopregnanolone

TABLE XXII

Experiment 3

Specific activities of isolated steroids

Compound	cpm ^{3}H/mg
isopregnanolone	5.3 x $10^{\cdot}$
3α,16α-dihydroxy-5α-pregnan-20-one	5.6 x 10^3
3α,16α-dihydroxy-5β-pregnan-20-one	6.8 x 10^4
5β-pregnan-3α,16α,20α-triol	4.2 x 10^4
16α-hydroxy-progesterone	6.2 x 10^4

and 16α-hydroxy-progesterone were calculated after acetylation with 1-^{14}C-acetic anhydride and crystallization with carrier. The difference between the two values is significant, although it should be noted that a second derivative was made only in the case of 16α-hydroxy-progesterone. Derivative formation and recrystallization provides a considerably greater degree of confidence that radiochemical homogeneity has been achieved. Lack of carrier steroids prevented similar measurements of the reduced metabolites. Direct measurement of specific activity was less precise because of the small amounts of the steroids isolated. The ten-fold difference in the specific activities of 3α,16α-dihydroxy-5α-pregnan-20-one and the other compounds is striking, and is without obvious explanation. It is possible that this metabolite is derived largely from a precursor other than 16α-hydroxy-progesterone. It

is unlikely that a difference of this magnitude can be explained on the basis of failure to achieve radiochemical homogeneity.

Pregnanolone, found in an early fraction from the initial silica gel column, contained no radioactivity. If isopregnanolone is derived from 16-dehydroprogesterone (40) then the absence of isotope in pregnanolone indicates a high degree of specificity in the reduction of the precursor, as suggested by Calvin and Lieberman.

EXPERIMENT 4

This experiment was designed to determine whether 16α-hydroxy-progesterone was present in the urine of a normal male, and, if so, in what form it was excreted. Extraction of the urine before hydrolysis of steroid conjugates yielded negligible amounts of radioactivity. The sulphate fraction contained a significant amount of tritium, representing at least two compounds. The more polar of the two, not completely identified, had chromatographic mobilities comparable to those of isopregnanolone. We concluded that the sulphate fraction contained little or no 16α-hydroxy-progesterone. The glucosiduronidate fraction, however, yielded the latter compound. The final fraction, just prior to acetylation with 1-^{14}C-acetic anhydride, contained 47,800

cpm. Assuming that all of this radioactivity was in the form of 16α-hydroxy-progesterone, and knowing its specific activity (1.7×10^6 cpm/mg) we can calculate a maximum recovery of 0.028 mg of 16α-hydroxy-progesterone. The recovery from pregnancy urine was 45-fold greater.

EXPERIMENTS 5 AND 6

These studies were planned to provide information about the precursors of 16α-hydroxy-progesterone in pregnancy. In Experiment 5, the subject was given 4-^{14}C-progesterone and 7α-^{3}H-16α-hydroxy-progesterone intravenously. In view of the large amounts of progesterone made in pregnancy, and the ability of the human female to hydroxylate at least one steroid in the 16α position (20), it seemed reasonable to suppose that circulating progesterone could serve as a precursor of urinary 16α-hydroxy-progesterone. However, when the latter compound was isolated, it contained no detectable carbon-14. We concluded from this that little or none of the urinary 16α-hydroxy-progesterone was produced by hepatic hydroxylation of progesterone from the maternal circulation. The short half-life of progesterone in the circulation (63) may, in part, explain the observation. It is also clear that little or none of the urinary 16α-hydroxy-progesterone was produced by foetal hydroxylation of maternal progesterone.

In Experiment 6, the subject was given 7α-^{3}H-16α-hydroxy-pregnenolone and 4-^{14}C-16α-hydroxy-progesterone. The 16α-hydroxy-progesterone isolated from urine contained both tritium and carbon-14, demonstrating that circulating 16α-hydroxy-pregnenolone was converted to 16α-hydroxy-progesterone. However, the ratio of tritium to carbon-14 in the isolated compound was higher than that of the injected mixture. We were forced to conclude that of the two injected compounds, 16α-hydroxy-pregnenolone was a better precursor of urinary 16α-hydroxy-progesterone than was 16α-hydroxy-progesterone itself. This rather surprising finding is not without precedence. Korenman and Lipsett (64) have recently published data demonstrating that testosterone may be formed in the liver from androstendione, and then be conjugated and excreted, without becoming part of the circulating pool of free testosterone. It is quite clear that in our experiment the labelled 16α-hydroxy-pregnenolone did not contribute to urinary 16α-hydroxy-progesterone by exclusive transformation to free circulating 16 -hydroxy-progesterone, for in these circumstances the ratio of tritium to carbon-14 in the urinary steroid could not exceed that of the injected compounds. We assume that 16α-hydroxy-progesterone was excreted as a glucosiduronidate as it was in the normal male. The validity of the latter assumption in no way affects the conclusions drawn thus far.

Figure 21 illustrates a set of pathways that account for the tritium and carbon-14 content of the urinary 16α-hydroxy-progesterone. The four compartments, A, B, C and X represent pools of steroid. The compounds in A and B, at least, are circulating, but we do not know if the pools exist naturally or were created by the injection. We have not demonstrated the presence of 16α-hydroxy-progesterone in blood, or of 16α-hydroxy-pregnenolone in blood or urine, although the latter compound has been found in the urine of the normal newborn (19). Compartment C is real, for we have isolated 16α-hydroxy-progesterone glucosiduronidate. Compartment X is required to provide a pathway from A to C not involving free circulating 16α-hydroxy-progesterone. It might represent, for example, a single tissue in which both oxidation and conjugation take place. The scheme is, of course, speculative and in reality the pathways may be much more complex. We can not say, for instance, that the transformations of 16α-hydroxy-pregnenolone take place in a single compartment, nor do we know from which compartments the metabolites of 16α-hydroxy-progesterone are derived. It is clear however that simple transformation of 16α-hydroxy-pregnenolone to free 16α-hydroxy-progesterone is an inadequate explanation of the data.

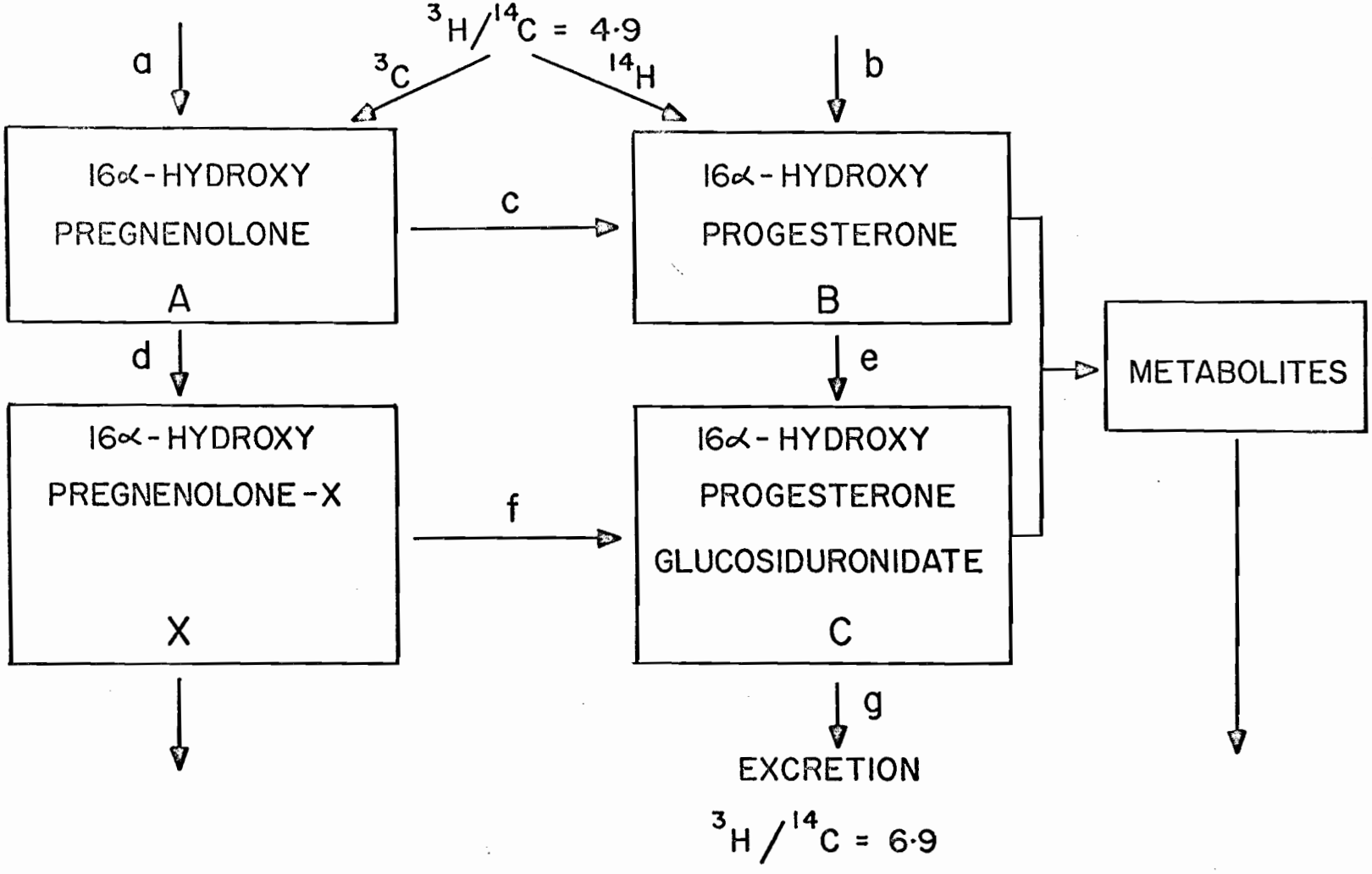

FIG. 21. EXPERIMENT 6.

PRODUCTION AND SIGNIFICANCE OF 16α-HYDROXY-PROGESTERONE

The experiments described provide little information about the site of production of 16α-hydroxy-progesterone. The adrenal cortex of the normal male is a likely source, in view of the evidence from *in vitro* studies discussed in the introduction. The problem of origins in pregnancy is more complex. If we make the reasonable assumption that the production of 16α-hydroxy-progesterone by the non-pregnant female is of the same order as that of the male, then it is clear that the pregnant subject makes increased amounts of the compound. Three possible sources are suggested by the available information, none of which are exclusive of the others. The maternal ovary and adrenal may secrete 16α-hydroxy-progesterone, presumably under the influence of some humoral factor. The foetus may be hydroxylating progesterone delivered to it by the placenta. It has been shown (68) that 16α-hydroxy-progesterone is a major metabolite of progesterone, when the latter is infused into the umbilical vein of the intact foetus. Finally, the foetus may be producing 16α-hydroxy- compounds *de novo* (59), that is, from precursors made in the foetal adrenal. The foetal adrenal is deficient in 3β-ol dehydrogenase activity, and presumably most of its products are 3β-ol-Δ^5 compounds. However the placenta is rich in 3β-ol dehydrogenase activity (59) and might serve to convert 16α-hydroxy-pregnenolone to

16α-hydroxy-progesterone.

The significance of 16α-hydroxylation is even less clear. It is known that 16α-hydroxylation reduces the mineralocorticoid effects of a number of steroids (65). Neher found that 3β,16α-dihydroxy-5α-pregnan-20-one caused sodium loss in certain assays in the rat (38). However Coppage and Liddle (66) were not able to demonstrate such an effect in the intact human, even with very large doses. Wettstein made a similar observation regarding the effects of 3α,16α-dihydroxy-5β-pregnan-20-one on the excretion of sodium by the rat (14), but to date the compound has not been studied in the human subject. Uete and Venning studied the effects of 16α-hydroxy-progesterone and 16α-hydroxy-pregnenolone on the response of adrenalectomized rats to aldosterone (67). They found that 16α-hydroxy-progesterone reduced the sodium retaining effect of aldosterone, but did not change the effect of aldosterone on potassium excretion. They noted that 16α-hydroxy progesterone was less effective than progesterone in antagonizing the action of aldosterone. There was no evidence that 16α-hydroxy-pregnenolone in any way influenced the action of aldosterone.

To sum up, 16α-hydroxy steroids may serve in pregnancy as precursors of oestriol, a compound which has no known biological function. Hydroxylation of sodium-retaining steroids in the 16α-position reduces or eliminates

their sodium-retaining potency, and 16α-hydroxylation of progesterone reduces its ability to antagonize the electrolyte effects of aldosterone.

The production rate of 16α-hydroxy-progesterone of the subjects of Experiments 3 and 4 may be calculated from the specific activity of the urinary 16α-hydroxy-progesterone, using the simple expression for isotope dilution (69).

$$P.R. = \frac{\text{cpm injected}}{\text{S.A. metabolite} \times t}$$

where P.R. = production rate

S.A. = specific activity

t = time of urine collection.

The rates so calculated for the male and pregnant female subjects are 0.4 mg and 16.3 mg per day respectively.

However, this method of calculation of production rates is valid only if the urinary steroid is uniquely derived from the pool in which production occurs. If the presence of 16α-hydroxy-pregnenolone in the blood or urine of the pregnant subject were demonstrated, then the data of Experiment 6 would invalidate the calculation.

SUMMARY

We have prepared isotopically labelled 16α-hydroxy-progesterone and 16α-hydroxy-pregnenolone by microbiological hydroxylation of appropriate steroid precursors. From the urine of a normal male given 4-^{14}C-16α-hydroxy-progesterone by intravenous injection, and unlabelled 16α-hydroxy-progesterone by mouth, we have isolated labelled 3α-hydroxy-5β,17α-pregnan-20-one, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one, 5α-pregnan-3α,16α,20α-triol and 5β-pregnan-3α,16α,20α-triol. From the urine of a pregnant subject given 7α-^{3}H-16α-hydroxy-progesterone intravenously we have isolated labelled 3α-hydroxy-5β-17α-pregnan-20-one, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one, 5β-pregnan-3α,16α,20α-triol, and 16α-hydroxy-progesterone. It has been shown that urinary 16α-hydroxy-progesterone of pregnancy may be derived in part from 16α-hydroxy-pregnenolone, but not from circulating maternal progesterone. The transformation of 16α-hydroxy-pregnenolone to urinary 16α-hydroxy-progesterone involves a pathway other than direct transformation to free circulating 16α-hydroxy-progesterone. The presence of 16α-hydroxy-progesterone in the urine of a normal male

given only a tracer dose of labelled 16α-hydroxy-progesterone has been demonstrated. In the latter study, 16-hydroxy-progesterone was excreted as a glucosiduronidate.

We have demonstrated then, that 16α-hydroxy-progesterone is a normal constituent of human urine, that, in the male at least, it is excreted as the glucosiduronidate, and that it is present in greater amounts in pregnancy. The urinary 16α-hydroxy-progesterone of pregnancy may be derived from 16α-hydroxy-pregnenolone, but not from circulating progesterone. Finally, we have shown that 3α-hydroxy-5β,17α-pregnan-20-one, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan, 5α-pregnan-3α,16α,20α-triol and 5β-pregnan-3α,16α,20α-triol are metabolites of 16α-hydroxy-progesterone.

CLAIMS TO ORIGINAL RESEARCH

1. Development of a novel method of synthesizing labelled 16α-hydroxy-steroids.

2. Demonstration that isopregnanolone, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one, 5α-pregnan-3α,16α,20α-triol and 5β-pregnan-3α,16α,20α-triol are metabolites of exogenous 16α-hydroxy-progesterone.

3. Isolation of 16α-hydroxy progesterone from the urine of a pregnant subject, and demonstration of its presence in the urine of a normal male.

4. Demonstration that circulating 16α-hydroxy-pregnenolone is converted to urinary 16α-hydroxy-progesterone, and that circulating progesterone is not so converted.

5. Demonstration, in pregnancy, of the origin of 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one and 5β-pregnan-3α,16α,20α-triol all of which are derived, in part at least, from 16α-hydroxy-progesterone.

6. Determination of the production rate of 16α-hydroxy-progesterone in a pregnant subject and in a normal male subject.

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