Pregnenolone and 15α-Hydroxysteroids in Pregnancy.

Frank Z. Stanczyk

ABSTRACT

Two different sets of experiments are described in this thesis. When labeled pregnenolone was injected into the umbilical vein of a Rhesus monkey with the fetus in utero, the substrate was not metabolized to any extent in any of the tissues examined except in the liver. 3β-Hydroxy-5α-pregnan-20-one was isolated from the fetal heart, intestine, liver and from the placenta. Also, the conversion of pregnenolone to progesterone by the fetal liver was demonstrated. In another set of experiments, non-labeled and labeled 15α-hydroxydehydroisoandrosterone was synthesized chemically from 15α-hydroxyandrostenedione. After the isolation of 1.76 and 3.20 μg/day of 15α-hydroxydehydroisoandrosterone from human late pregnancy urine, the metabolism of this compound together with 15α-hydroxyandrostenedione was studied in pregnant subjects. Following the intravenous injection of a mixture of ^{3}H-15α-hydroxyandrostenedione and ^{14}C-15α-hydroxydehydroisoandrosterone into two subjects in the third trimester of pregnancy, it was shown that the latter precursor was converted to urinary 15α-hydroxyandrostenedione, 15α-hydroxyestradiol and 15α-hydroxyestriol. However, 15α-hydroxyandrostenedione was a more efficient precursor of urinary 15α-hydroxyestradiol and 15α-hydroxyestriol than 15α-hydroxydehydroisoandrosterone. These findings were confirmed when the same substrates were introduced into two fetuses in utero during transfusion for erythroblastosis fetalis.

STUDIES ON THE METABOLISM OF PREGNENOLONE IN THE PREGNANT RHESUS MONKEY AND 15α-HYDROXYLATED STEROIDS IN HUMAN PREGNANCY

by

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TRIVIAL AND SYSTEMATIC NAMES

Trivial Name	Systematic Name
estrone	3-hydroxyestra-1,3,5(10)-trien-17-one
estrone sulfate	3-sulfoxyestra-1,3,5(10)-trien-17-one
15α-hydroxyestrone	3,15α-dihydroxyestra-1,3,5(10)-trien-17-one
15β-hydroxyestrone	3,15β-dihydroxyestra-1,3,5(10)-trien-17-one
16α-hydroxyestrone	3,16α-dihydroxyestra-1,3,5(10)-trien-17-one
estradiol	estra-1,3,5(10)-triene-3,17β-diol
15α-hydroxyestradiol	estra-1,3,5(10)-triene-3,15α,17β-triol
15β-hydroxyestradiol	estra-1,3,5(10)-triene-3,15β,17β-triol
estriol	estra-1,3,5(10)-triene-3,16α,17β-triol
15α-hydroxyestriol	estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol
15α-hydroxyestradiol triacetate	3,15α,17β-triacetoxyestra -1,3,5(10)-triene
15α-hydroxyestriol tetraacetate	3,15α,16α,17β-tetraacetoxyestra-1,3,5(10)-triene
androstenedione	androst-4-ene-3,17-dione
11β-hydroxyandrostenedione	11β-hydroxyandrost-4-ene-3,17-dione
15α-hydroxyandrostenedione	15α-hydroxyandrost-4-ene-3,17-dione
15α-hydroxyandrostenedione enol acetate	3,15α-diacetoxyandrost-3,5-dien-17-one
16α-hydroxyandrostenedione	16α-hydroxyandrost-4-ene-3,17-dione
testosterone	17β-hydroxyandrost-4-en-3-one
15α-hydroxytestosterone	15α,17β-dihydroxyandrost-4-en-3-one
15α-acetoxytestosterone	15α-acetoxy-17β-hydroxyandrost-4-en-3-one
15α-hydroxytestosterone diacetate	15α,17β-diacetoxyandrost-4-en-3-one
dehydroisoandrosterone	3β-hydroxyandrost-5-en-17-one

Trivial Name	Systematic Name
dehydroisoandrosterone sulfate	3β-sulfoxyandrost-5-en-17-one
15α-hydroxydehydroisoandrosterone	3β,15α-dihydroxyandrost-5-en-17-one
15α-hydroxydehydroisoandrosterone diacetate	3β,15α-diacetoxyandrost-5-en-17-one
16α-hydroxydehydroisoandrosterone	3β,16α-dihydroxyandrost-5-en-17-one
16α-hydroxydehydroisoandrosterone-3-sulfate	16α-hydroxy-3β-sulfoxyandrost-5-en-17-one
progesterone	pregn-4-ene-3,20-dione
5α-pregnanedione	5α-pregnane-3,20-dione
5β-pregnanedione	5β-pregnane-3,20-dione
20α-dihydroprogesterone	20α-hydroxypregn-4-en-3-one
20β-dihydroprogesterone	20β-hydroxypregn-4-en-3-one
6β-hydroxyprogesterone	6β-hydroxypregn-4-ene-3,20-dione
15α-hydroxyprogesterone	15α-hydroxypregn-4-ene-3,20-dione
17α-hydroxyprogesterone	17α-hydroxypregn-4-ene-3,20-dione
deoxycorticosterone	21-hydroxypregn-4-ene-3,20-dione
deoxycorticosterone acetate, DOCA	21-acetoxypregn-4-ene-3,20-dione
deoxycorticosterone sulfate	21-sulfoxypregn-4-ene-3,20-dione
cortisol	11β,17α,21-trihydroxypregn-4-ene-3,20-dione
pregnanolone	3α-hydroxy-5β-pregnan-20-one
pregnanediol	5β-pregnane-3α,20α-diol
pregnenolone	3β-hydroxypregn-5-en-20-one
pregnenolone sulfate	3β-sulfoxypregn-5-en-20-one
20α-dihydropregnenolone	pregn-5-ene-3β,20α-diol
20α-dihydropregnenolone-3-sulfate	3β-sulfoxypregn-5-en-20α-ol

Trivial Name	Systematic Name
15α-hydroxypregnenolone	3β,15α-dihydroxypregn-5-en-20-one
16α-hydroxypregnenolone	3β,16α-dihydroxypregn-5-en-20-one
16α-hydroxypregnenolone-3-sulfate	16α-hydroxy-3β-sulfoxypregn-5-en-20-one
17α-hydroxypregnenolone	3β,17α-dihydroxypregn-5-en-20-one
17α-hydroxypregnenolone-3-acetate	3β-acetoxy-17α-hydroxypregn-5-en-20-one
17α-hydroxypregnenolone-3-sulfate	17α-hydroxy-3β-sulfoxypregn-5-en-20-one
21-hydroxypregnenolone	3β,21-dihydroxypregn-5-en-20-one
21-hydroxypregnenolone diacetate	3β,21-diacetoxypregn-5-en-20-one
cholesterol	cholest-5-en-3β-ol

ABBREVIATIONS

mm	millimeter
cm	centimeter
ml	milliliter
µg	microgram
mg	milligram
g	gram
M	mole
µM	micromole
mM	millimole
N	normal
V/V	volume/volume
W/V	weight/volume
µc	microcurie
mc	millicurie
dpm	disintegrations per minute
mp	melting point
NAD	nicotinamide-adenine dinucleotide
DDQ	2,3-dichloro-5,6-dicyano-p-benzoquinone
HBV	hold-back volume
R_f	the ratio of the distance traversed by a given compound over that traversed by the solvent mixture
NMR	nuclear magnetic resonance
δ	chemical shift in parts per million
ppm	parts per million
M^+	parent molecular ion
IUT	intrauterine transfusion

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INTRODUCTION

This thesis will present data on two different sets of experiments. One portion of the thesis will deal with the metabolism of pregnenolone by the placenta and fetal tissues of the Rhesus monkey, whereas the second part will be concerned with the chemical synthesis and isolation of 15α-hydroxydehydroisoandrosterone, as well as with the metabolism of 15α-hydroxyandrostenedione and 15α-hydroxydehydroisoandrosterone in pregnant subjects. Current knowledge regarding the nature, formation and metabolism of steroids in the pregnant Rhesus monkey, and the isolation, formation and metabolism of 15α-hydroxysteroids in human pregnancy will be reviewed.

Nature of Steroids in Pregnant Rhesus Monkey

Prior to 1961, it was often assumed, for several reasons, that the endocrine changes occurring during pregnancy in Rhesus monkeys were very similar to those taking place in pregnant women. For example, a number of investigators had demonstrated that in the Rhesus, like in the human, ovariectomy (1,2,3,4,5) or hypophysectomy (6,7) early in pregnancy did not interfere with the subsequent course of gestation. In addition, it had been shown that the Rhesus monkey, like the human female, excreted chorionic gonadotropin in the urine during pregnancy, although in the Rhesus the urinary excretion of this hormone dropped early in pregnancy (8,9). Furthermore, studies in Rhesus monkeys had shown that, as in women, estrogenic activity in the urine increased during pregnancy (10).

It soon became apparent that the endocrine changes occurring during pregnancy in the Rhesus monkey were not nearly as conspicuous as those seen during pregnancy in women. In 1961, Short and Eckstein (11)

reported a striking difference in the concentrations of placental progesterone between the two species, the levels in the Rhesus being 30-40 times lower than in the human. They also found that the progesterone levels in the peripheral blood, as well as in the uterine vein and umbilical cord blood, were considerably lower than those measured in the comparable gestation period of pregnant women. These investigators concluded that as far as the concentration of progesterone was concerned, the Rhesus seemed to be more closely related to the domestic animals than to the higher primates. In view of these findings, it is not surprising that pregnanediol, the most abundant metabolite of progesterone in human pregnancy urine, is excreted only in very small amounts in the urine of the pregnant Rhesus. This metabolite was detected by Chamberlain et al (12) using gas-liquid chromatographic procedures.

Another striking difference that has been observed between the two species is in the urinary excretion pattern of estrogens. Hopper and Tullner (13) provided evidence for the presence of estrone, estradiol and estriol in the pregnancy urine of the Rhesus by means of gas-liquid chromatography. These investigators showed that, as in pregnant women, there was a progressive increase in the excretion of the three estrogens with advancing gestation. They also observed that the excretion of estrone exceeded that of the combined urinary excretion of estradiol and estriol in each trimester of pregnancy in Rhesus monkeys, and that the marked increase in urinary estriol during human pregnancy was not observed in this species. Some of these observations were later confirmed by Liskowski et al (14).

Finally, there is a difference in the concentrations of total plasma cholesterol found in human pregnancy and the pregnant Rhesus.

Wolf et al (15) reported a decrease in the plasma concentration of total cholesterol during pregnancy in Rhesus monkeys. The decline was observed within one month following conception and continued until the twelfth week of gestation, after which time the plasma cholesterol concentration remained constant until one week following parturition, when a return to the nonpregnant level was observed. At three weeks postpartum, the plasma cholesterol concentration was the same as that found prior to conception. On the other hand, in human pregnancy there is a slight decrease in plasma cholesterol during the first trimester of pregnancy, which is followed by a progressive increase, and the peak value occurs at, or near, parturition (16).

Formation and Metabolism of Steroids in Pregnant Rhesus Monkey

Despite the extensive use of the Rhesus monkey for the study of reproductive physiology, very little is known regarding the formation and metabolism of steroid hormones in this species (17). Several in vitro studies have been reported dealing with the incubation of placental tissue or fetal tissues from the Rhesus with labeled precursors. Ainsworth et al (18) incubated the 800 x g supernatant fraction of Rhesus placental tissue (third trimester) with ^{3}H-dehydroisoandrosterone and ^{14}C-androstenedione and isolated estrone and estradiol containing both labels. In a second experiment, these investigators incubated the supernatant fraction with ^{3}H-pregnenolone and ^{14}C-progesterone and isolated the following metabolites containing both labels: progesterone, 20α-dihydroprogesterone, 6β-hydroxyprogesterone, 3β-hydroxy-5α-pregnan-20-one, 5α-pregnane-3β,20α-diol and 5α-pregnanedione.

The capacity of the fetal gonad of the Rhesus to produce androgens was studied by Resko (19). He incubated first trimester fetal testes with ^{14}C-pregnenolone and demonstrated the conversion of this substrate to androstenedione and testosterone. Measurement of testosterone and androstenedione in umbilical artery plasma showed more testosterone in plasma from male fetuses, whereas the androstenedione concentrations did not differ with fetal sex. Greater quantities of testosterone were also found in the peripheral plasma of mothers with male fetuses. Incubation of fetal ovaries with ^{14}C-pregnenolone indicated that this tissue was relatively quiescent in regard to its ability to synthesize testosterone and androstenedione during the first half of pregnancy.

Gorwill et al (20) incubated homogenates of mid-trimester fetal Rhesus monkey adrenals simultaneously with ^{14}C-pregnenolone and ^{3}H-pregnenolone sulfate and reported the conversion of both substrates to 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroisoandrosterone, androstenedione, 11β-hydroxyandrostenedione, cortisol, 17α-hydroxypregnenolone-3-sulfate and dehydroisoandrosterone sulfate. In addition, these authors also demonstrated the conversion of pregnenolone to progesterone.

Heinrichs and Colas (21) incubated liver microsomes prepared from fetal, newborn and adult Rhesus monkeys with dehydroisoandrosterone in order to determine the extent of 16α-hydroxylation by this tissue. They found the lowest 16α-hydroxylase activity in the microsomes from the fetal liver, higher activity in the liver of the newborn and the highest activity in the adult liver. The authors concluded that the low rate of hepatic 16α-hydroxylation in monkey fetuses was consistent with the small urinary excretion of estriol by this species during pregnancy.

There are only two reports dealing with the in vivo metabolism of steroids in the Rhesus during pregnancy. Snyder et al (22) perfused mid-term placentas in situ with ^{14}C-dehydroisoandrosterone and ^{3}H-dehydroisoandrosterone sulfate and found a very limited conversion of both substrates to androstenedione and to estrogens. There was, however, extensive hydrolysis of the sulfate. Leung and Solomon (23) injected ^{14}C-progesterone into the umbilical vein of the Rhesus monkey in the third trimester of pregnancy with the fetus in utero and isolated the following metabolites in the unconjugated form from the fetal tissues and placenta: 5α-pregnanedione from the placenta; pregnanolone from the liver and intestine; 3β-hydroxy-5α-pregnan-20-one from the testes, lung and placenta; 3α-hydroxy-5β-pregnan-20-one and pregnanediol from the liver; 20α-dihydroprogesterone from the intestine and lung; 17α-hydroxyprogesterone from the adrenal and testes; androstenedione from the adrenal. Progesterone was found in every tissue examined, but only small amounts of this substrate were found in the fetal liver and placenta. The authors concluded that progesterone was mainly metabolized to reduced products by the placenta and fetal tissues of the pregnant Rhesus monkey, and that the only hydroxylated product formed was 17α-hydroxyprogesterone.

Limitations in the study of steroid metabolism in human fetuses have created a need for a nonhuman primate species to serve as a model for the human feto-placental unit. After reviewing the literature, it was obvious that very little was known regarding the metabolism of steroids in the Rhesus monkey during pregnancy. Since such data are essential for evaluating the suitability of the Rhesus as a model for the human feto-placental unit, it was of interest to us to continue the studies on the

Rhesus begun by Leung and Solomon (23), and to investigate the metabolism of pregnenolone in the fetal tissues and placenta of this species.

Isolation of 15α-Hydroxylated Steroids From Human Pregnancy Urine

During the past few years, a number of 15α-hydroxylated steroids, both phenolic and neutral, have been isolated from human pregnancy urine. These steroids, along with their estimated rates of excretion in human late pregnancy urine, are listed in Table 1. The first report of the isolation of a 15α-hydroxysteroid from human urine was published in 1965 by Knuppen et al (24), who found 15α-hydroxyestrone in late pregnancy urine. The authors reported the concentration of this steroid in pregnancy urine to be approximately the same as that of 16α-hydroxyestrone. Two years later, Zucconi et al (25) identified 15α-hydroxyestriol in pregnancy urine. These investigators used 200 liters of late pregnancy urine to isolate sufficient amounts of the tetrol for establishing its structure. Previously, this steroid had been tentatively identified by Hagen et al (26) in the urine of malformed infants. Also, Gurpide et al (27) had isolated the tetrol from pregnancy urine and assigned it the structure of 15α-hydroxyestriol. In the same year, two different groups of investigators reported the isolation of 15α-hydroxyestradiol from human pregnancy urine. Lisboa et al (29) isolated 5 mg of this steroid from a pool of 100 liters of acid-hydrolyzed late pregnancy urine, and Knuppen and Breuer (30) identified 15α-hydroxyestradiol in extracts of enzymatically hydrolyzed late pregnancy urine. On the other hand, using gas-liquid chromatography, Luukkainen and Adlercreutz (31) could not detect the presence of 15α-hydroxyestradiol in urine collected during the second trimester of gestation.

Table 1

15α-Hydroxylated Steroids Isolated From Human Pregnancy Urine

Steroid	Approximate Amount (μg/day)	Reference
15α-Hydroxyestrone	-	24
15α-Hydroxyestriol	100-520	25,27,28
15α-Hydroxyestradiol	50-100	29,30
15α-Hydroxyprogesterone	28-34	32
15α-Hydroxyandrostenedione	4.4-5.2	34
15α-Hydroxytestosterone	0.48-0.64	34
3β,15α-Dihydroxy-5α-pregnan-20-one	170	35
3β,15α-Dihydroxy-5β-pregnan-20-one	106	35
15α-Hydroxypregnenolone	1-2	36

The first report of the isolation of a neutral 15α-hydroxylated steroid from human pregnancy urine was published in 1967 by Giannopoulos and Solomon (32), who isolated crystalline 15α-hydroxyprogesterone from late pregnancy urine. Using isotopic dilution techniques, these authors found that the urinary excretion rate of 15α-hydroxyprogesterone in the third trimester of pregnancy was 28-34 μg/day. Lesser amounts (5-6 μg/day) of this steroid were detected in second trimester pregnancy urine and negligible quantities were found in urine collected during the first trimester of gestation (33). Furthermore, the same investigators succeeded in detecting small amounts of 15α-hydroxyandrostenedione and 15α-hydroxytestosterone in human late pregnancy urine (34). In 1970, Jänne and Vihko (35) reported the isolation of two 15α-hydroxylated C_{21} steroids, namely, 3β,15α-dihydroxy-5α-pregnan-20-one and 3β,15α-dihydroxy-5β-pregnan-20-one. These compounds were identified and measured in human late pregnancy urine by gas-liquid chromatography and gas chromatography-mass spectrometry, and were present in the urine as monosulfates. Very recently, Stern and Solomon (36), using isotopic dilution techniques, detected small amounts of 15α-hydroxypregnenolone in human late pregnancy urine.

Formation of 15α-Hydroxylated Estrogens in Pregnancy

Since the isolation of 15α-hydroxyestrogens from human pregnancy urine, a great deal of attention has focused on their formation during pregnancy. It soon became apparent from *in vivo* studies that the principal site of 15α-hydroxyestrogen formation in the pregnant woman was the feto-placental unit and that these steroids can be formed by 15α-hydroxylation of estrogens. Schwers et al (37) perfused previable human fetuses with ^{3}H-estrone and ^{14}C-estrone sulfate and, in a separate study (38), with

^{3}H-estrone and ^{14}C-estradiol and showed that all of the precursors were incorporated into 15α-hydroxyestradiol, which was isolated from the conjugated fraction of the fetal liver. In yet another study by the same investigators (39), a conjugated form of 15α-hydroxyestradiol was identified in the midterm fetal liver, following the injection of labeled estrone and estradiol into the intact feto-placental unit in situ. In all the experiments just described, no 15α-hydroxyestradiol was detected in any of the other fetal tissues examined. Mancuso et al (40) perfused previable human fetuses with labeled androstenedione and testosterone and demonstrated that both neutral precursors were converted to 15α-hydroxyestradiol isolated from the fetal liver, but they were unable to detect labeled 15α-hydroxyandrostenedione or 15α-hydroxytestosterone. On the basis of these results, the authors suggested that the formation of 15α-hydroxyestrogens in the human fetus at midterm occurred in the liver and proceeded only via the 15α-hydroxylation of phenolic precursors. In a series of studies, Gurpide et al (27) injected ^{3}H-estradiol into the amniotic cavity of two pregnant women (second and third trimesters of pregnancy) and, in another experiment, into the peritoneal cavity of a midterm fetus in utero during a blood transfusion for Rh incompatibility. In each of these experiments, ^{14}C-estradiol was injected into a maternal peripheral vein simultaneously with the administration of the tritiated tracer. The authors found that 15α-hydroxyestriol was the major metabolite of estradiol and that the formation of the tetrol occurred predominantly in the fetus. Subsequently, Schwers et al (41) demonstrated that when a mixture of ^{3}H-estriol and ^{14}C-estradiol was administered intra-amniotically to a subject in the 14th week of gestation, both tracers were incorporated

into urinary 15α-hydroxyestriol. Furthermore, on the basis of their data, the authors suggested that the conversion of estradiol to the tetrol occurred only partially via estriol. Thus, in all these studies it appeared that 15α-hydroxylation occurred after aromatization in the feto-placental unit.

Following the isolation of 15α-hydroxyandrostenedione and 15α-hydroxytestosterone from late pregnancy urine, attention was turned to the question of whether 15α-hydroxyestrogens can also be formed by the placental aromatization of 15α-hydroxylated neutral precursors. Several types of experimental approaches showed conclusively that not only can 15α-hydroxylated neutral steroids be aromatized, but also serve as good substrates for the formation of 15α-hydroxyestrogens in pregnancy. In an *in vitro* study, Stern et al (42) incubated labeled 15α-hydroxyandrostenedione with a 10,000 x g fraction prepared from human term placental tissue and obtained 29-39% conversion of the substrate to 15α-hydroxyestradiol. These results were confirmed by Meeker et al (43), using an *in vivo* experimental approach. This study involved the perfusion of the placenta *in situ* with labeled 15α-hydroxyandrostenedione at the time of Caesarean section and showed that the difference in the results obtained by the *in vitro* and *in vivo* methods was essentially quantitative in nature. From these data, it was concluded that the placenta can readily aromatize 15α-hydroxyandrostenedione to 15α-hydroxyestradiol and that this may be the pathway which is operative during late pregnancy in the formation of the 15α-hydroxyestrogens, in analogy with the formation of the estriol from 16α-hydroxylated neutral precursors (44,45). It is well known that a large fraction of estriol is derived by fetal 16α-hydroxylation of neutral precursors and their subsequent aromatization in the placenta.

Further evidence for the precursor role of C_{19} steroids in the formation of 15α-hydroxyestrogens, during the third trimester of pregnancy, was obtained from a number of in vivo experiments. YoungLai and Solomon (28), in a series of studies, injected pairs of the following ^{3}H-and ^{14}C-labeled precursors into the maternal circulation: dehydroisoandrosterone sulfate, 16α-hydroxydehydroisoandrosterone, 16α-hydroxydehydroisoandrosterone sulfate, 16α-hydroxyandrostenedione and 15α-hydroxyandrostenedione. All the substrates were converted to urinary 15α-hydroxyestriol, and dehydroisoandrosterone sulfate was found to be the best precursor of this metabolite. Furthermore, the authors concluded that 15α-hydroxyandrostenedione was a better precursor than 16α-hydroxyandrostenedione in the formation of the urinary tetrol. Finally, in two of the studies, it was shown that dehydroisoandrosterone sulfate and 15α-hydroxyandrostenedione were converted to urinary 15α-hydroxyestradiol, but 15α-hydroxyandrostenedione seemed to be the better precursor. An inherent objection to the type of approach used in the studies just described is lack of knowledge regarding the relative amounts of labeled substrates entering the feto-placental unit from the maternal circulation. In order to overrule this objection, YoungLai and Solomon (46) performed a study using an experimental design in which the labeled substrates were injected into the fetus during an intrauterine transfusion for erythroblastosis fetalis. Following the administration of a mixture of ^{3}H-dehydroisoandrosterone sulfate and ^{14}C-15α-hydroxyandrostenedione into the fetus, the authors demonstrated that 15α-hydroxyandrostenedione was a better precursor than dehydroisoandrosterone sulfate for both urinary 15α-hydroxyestradiol and 15α-hydroxyestriol. Their data also showed that 15α-hydroxylation was followed by 16α-hydroxylation in the

formation of 15α-hydroxyestriol, but it was uncertain whether 16α-hydroxylation occurred prior to, or after, aromatization. Recently, Schut and Solomon (47) injected ^{3}H-15α-hydroxyestradiol and C^{14}-estradiol into the maternal circulation of pregnant subjects and, in a second study, into the fetus during an intrauterine transfusion for erythroblastosis fetalis. The results showed that both 15α-hydroxyestradiol and estradiol were equally good precursors of urinary 15α-hydroxyestriol and, consequently, no conclusion could be drawn as to the order of 15α-and 16α-hydroxylations of phenolic precursors. Furthermore, the data showed that 15α-hydroxyestriol was derived from estradiol only partially via 15α-hydroxyestradiol. In yet another experiment, the same authors administered ^{3}H-15α-hydroxyandrostenedione and ^{14}C-estradiol into the peritoneal cavity of a fetus in utero and demonstrated that, of the two substrates, 15α-hydroxyandrostenedione was the better precursor of urinary 15α-hydroxyestradiol, whereas estradiol was shown to be the better precursor of urinary 15α-hydroxyestriol. In addition, 15α-hydroxyandrostenedione was found to be a better precursor of urinary 15α-hydroxyestrone than estradiol. It is obvious from all these studies that further work is necessary to elucidate the exact pathways leading to the formation of urinary 15α-hydroxyestradiol and 15α-hydroxyestriol.

Formation and Metabolism of 15α-Hydroxysteroids in Human Adults

Despite the fact that 15α-hydroxylation is regarded as a metabolic pathway unique to the fetus, there are indications that this reaction may also occur in the human adult. The in vitro 15α-hydroxylation of estrogens has been observed in the adult liver (48) and adrenals (49). Also, Jirku et al (50) isolated small amounts of 15α-hydroxyestrone from the sulfate fraction of bile, following the intravenous injection of ^{3}H-estrone

sulfate into a non-pregnant female. On the other hand, Giannopoulos et al (33) failed to detect 15α-hydroxyprogesterone in the urine of non-pregnant females and, in another study, showed that the conversion of progesterone to 15α-hydroxyprogesterone occurred during late pregnancy in the fetoplacental unit but not in the maternal circulation. Furthermore, as mentioned previously, Gurpide et al (27) demonstrated that the formation of 15α-hydroxyestriol from estradiol took place in the fetus but not in the mother. Thus, from these data, it appears that 15α-hydroxylation is quantitatively of minor importance in the human adult.

There have been only a few reports on the metabolism of 15α-hydroxysteroids in man. Knuppen et al (51) studied the metabolism of 15α-hydroxy-and 15-ketoestrogens in vitro using human liver slices and isolated a number of products including 15β-hydroxyestrone and 15β-hydroxyestradiol. Giannopoulos et al (52) studied the metabolism of 15α-hydroxyprogesterone in pregnant and non-pregnant subjects and isolated the following urinary metabolites: 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 3β,15α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,15α,20β-triol and 5β-pregnane-3α,15α,20β-triol. These authors concluded that the 15α-hydroxyl group partially inhibited the reduction of the Δ^4-3-ketone and the C-20 ketone, leading to an increased ratio of 5α: 5β-reduced urinary metabolites, and directed the reduction of the C-20 ketone toward the 20β-alcohol.

After reviewing the literature, it was quite obvious that our knowledge of the formation and metabolism of 15α-hydroxysteroids in humans was scanty. Although 15α-hydroxyandrostenedione and 15α-hydroxy-testosterone were found in human pregnancy urine, there were no reports on

the isolation or attempted isolation of any 15α-hydroxylated Δ^5-3β-hydroxy C_{19} compounds from human sources. It seemed that the isolation of such steroids would be quite significant since they might provide some evidence of the origin of the corresponding Δ^4-3-ketonic steroids. Furthermore, the demonstration of the conversion of dehydroisoandrosterone sulfate and 15α-hydroxyandrostenedione to urinary 15α-hydroxyestradiol and 15α-hydroxyestriol led to the consideration that dehydroisoandrosterone may be 15α-hydroxylated and then aromatized to form 15α-hydroxyestrogens. These possibilities were the stimulus for the initiation of the present studies on the isolation of 15α-hydroxydehydroisoandrosterone from pregnancy urine and the possible precursor role of this compound for 15α-hydroxyandrostenedione and 15α-hydroxyestrogens in human pregnancy.

MATERIALS AND METHODS

Organic Solvents

With the exception of absolute alcohol (Gooderham and Worts Ltd.), glacial acetic acid (reagent grade, Fisher Scientific Co.), acetyl chloride (analyzed reagent, J.T. Baker Chemical Co., Phillipsburg, N.J.) and ethylene glycol (reagent grade, Fisher Scientific Co.), all solvents were distilled prior to use. The following solvents were distilled without any additional treatment:-

acetone (reagent grade, Fisher Scientific Co.)

n-butanol (practical grade, Distillation Products Industries, Rochester, N.Y.)

t-butanol (practical grade, Distillation Products Industries, Rochester, N.Y.)

dioxane (practical grade, Distillation Products Industries, Rochester, N.Y.

ethyl acetate (reagent grade, Fisher Scientific Co.)

ethylene dichloride (reagent grade, Fisher Scientific Co.)

n-hexane (reagent grade, Fisher Scientific Co.)

iso-octane (practical grade, Distillation Products Industries, Rochester, N.Y.)

methanol (reagent grade, Fisher Scientific Co.)

methylcyclohexane (practical grade, Distillation Products Industries, Rochester, N.Y.)

methylene dichloride (reagent grade, Fisher Scientific Co.)

n-propanol (reagent grade, Fisher Scientific Co.)

toluene (reagent grade, Fisher Scientific Co.)

ligroin B (Skellysolve Oil Co., El Dorado, Kansas)

ligroin C (Skellysolve Oil Co., El Dorado, Kansas)

Other solvents were purified in a specific manner. Acetic anhydride (reagent grade, Fisher Scientific Co.) was distilled over fused sodium acetate, whereas pyridine was distilled over lumps of barium oxide. Both solvents were distilled under anhydrous conditions and were stored in desiccators. Benzene (reagent grade, Fisher Scientific Co.) and diethyl ether (analytical grade, Mallinckrodt) were distilled over potassium hydroxide pellets. The latter solvent and dioxane were stored at approximately 4°C. Tetrahydrofuran was refluxed over potassium hydroxide for at least two hours prior to distillation and was used immediately.

In the later stages of these studies, the following solvents were purchased from A & C American Chemicals, Ville St. Laurent, Que: acetone, benzene, n-butanol, t-butanol, ethyl acetate, diethyl ether, n-hexane, iso-octane, methanol, methylene chloride and toluene.

Certain organic solvents are known to form peroxides very readily. Thus, diethyl ether, dioxane and ethyl acetate were tested for the presence of peroxides prior to use. The peroxide test involved the addition of 1-2 ml of 1% (w/v) potassium iodide to approximately 5 ml of the organic solvent in a test tube. The appearance of a yellow color in the organic layer after the addition of a drop or two of 10% sulfuric acid indicated the presence of peroxides in the solvent.

Steroids

Non-labeled steroids used in these studies were obtained from several sources. Most of the steroids were purchased either from Ikapharm (Israel) or Mann Research Laboratories (New York) or Steraloids Inc. (New York). Two compounds, namely, 15α-hydroxyandrostenedione and 15α-hydroxyestradiol were gifts from Dr. C. Vezina, Ayerst Laboratories, Montreal, and

15α-hydroxyestriol was prepared by Mr. Herman Schut of this laboratory. All steroids used as carriers were crystallized several times prior to use and their identities were established by infrared spectroscopy.

Labeled steroids were purchased from New England Nuclear Corp., Boston, Mass. and were checked for purity as described in a separate section of this thesis. They were stored in benzene:methanol (4:1) at 4°C to minimize the danger of self-decomposition.

Cofactors and Enzymes

β-Nicotinamide-adenine dinucleotide and 3β-hydyroxysteroid dehydrogenase-Δ^5-isomerase (Pseudomonas testosteroni), Type I, were purchased from the Sigma Chemical Co., St. Louis, Mo. and were stored in a desiccator at 4°C. One mg of the enzyme oxidized 0.05 μM of testosterone per minute in the presence of βNAD at pH 8.9 and 25°C.

Infrared Spectroscopy

Infrared spectra were obtained using a Perkin-Elmer Model 221 Spectrophotometer. Samples were analyzed either as 1% solutions in CS_2 or as dispersions in KBr discs using 100-300 μg of steroid in approximately 20-25 mg of KBr. Smaller amounts (20-50 μg) of steroids were analyzed in KBr with the aid of a Perkin-Elmer 6X Microsampling Unit (Model 186-0011).

Measurement of Radioactivity

Samples of radioactive materials to be counted were prepared in the following manner. The samples were transferred to 5 dram vials (Wheaton Glass Co., Millville, N.J.), organic solvents in the vials were evaporated under nitrogen and the residues were dissolved in a suitable

counting solution. Samples soluble in toluene were dissolved in 15 ml of this solvent containing 0.3% (w/v) of 2,5-diphenyloxazole (PPO) and 0.1% (w/v) of 1,4-bis-(2,5-phenyloxazolyl)-benzene (POPOP). In the later stages of these studies, the scintillation solution was changed to toluene containing 0.4% (w/v) of Omnifluor (New England Nuclear Corp., Boston, Mass.). The efficiency of the Omnifluor solution was identical to that of the previous counting solution. On the other hand, samples not readily soluble in toluene were first dissolved in 2 ml of methanol and then 13 ml of scintillation solution was added. Urine samples were prepared for counting by mixing a 2 ml aliquot of the urine with 13 ml of Aquasol (New England Nuclear Corp., Boston, Mass.).

All samples were counted in a Model 3002 Packard Tri-Carb Liquid Scintillation Spectrophotometer. For single label counting, the discriminator settings were set at 50 to infinity and the gain of both the ^{3}H and ^{14}C channels was set at 60%. The efficiency of counting ^{3}H and ^{14}C at these settings was 35% and 85%, respectively. However, when samples were dissolved in methanol, the discriminator settings were set at 50 to infinity and the gain was set at 65% and 12% for the ^{3}H and ^{14}C channels, respectively. At these settings ^{3}H was counted with an efficiency of 30%, whereas the efficiency of counting ^{14}C was 80%. When methanol was present in the counting vials and ^{3}H and ^{14}C were counted simultaneously, the ^{3}H channel was set at 100% gain with discriminator settings at 50 and 850, and the ^{14}C channel settings were 16% for the gain and 225 and 1000 for the discriminators. At these settings, the counting efficiency of ^{3}H and ^{14}C was 27% and 57%, respectively. In order to obtain the maximum efficiency of counting, the settings given above were altered occasionally

during the course of these studies, however, the efficiencies of ^{3}H and ^{14}C varied only slightly from those shown. Samples were counted for a period of time sufficient to give a standard deviation of no more than 2% in the case of unquenched samples and 5% in the case of quenched samples.

Radioactivity in all samples was measured as dpm. ^{3}H-and/or ^{14}C-hexadecane standards (The Radiochemical Center, Amersham, Buckinghamshire, England) were counted with each set of samples, and the efficiency of counting of these standards was used to calculate the dpm for each sample. The standards were prepared in the following manner. Counting vials were washed with detergent, rinsed thoroughly with water and methanol and dried in an oven. After equilibrating at room temperature, the vials were weighed until a constant weight was obtained. Aliquots of ^{3}H-or ^{14}C-hexadecane were transferred to separate vials and dried over low heat under nitrogen. The vials were then desiccated for several hours and reweighed. This process of desiccating and weighing the vials was continued until a constant weight was obtained. The total dpm of each sample was computed from the weight of the hexadecane and its known specific activity.

In double label counting, the total ^{3}H and ^{14}C cpm were calculated by the discriminator ratio method of Okita et al (53) as modified by Ulick (54). The following equations were used:-

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

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

where N_1 = total counts in the first channel,

N_2 = total counts in the second channel,

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

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

The "a" and "b" ratios were determined by counting both the ^{3}H-and ^{14}C-hexadecane standards with each set of samples. Slight adjustments in the instrument settings were made occasionally to keep "a" and "b" ratios close to 0.01 and 5.0, respectively.

To correct for quenching, ^{3}H-toluene and ^{14}C-toluene were used as internal standards. Samples were recounted after the addition of each internal standard. Comparison of the increment of dpm in the sample with the actual number of dpm added, provided a ratio which was used to correct the original dpm of the quenched sample.

Celite Column Chromatography

Celite (#545,Johns-Manville) was neutralized prior to use. The Celite was first immersed in 50% (v/v) concentrated hydrochloric acid for 24 hours and was then washed thoroughly with tap water and distilled water until the washings were neutral. It was finally washed once with methanol and was dried in air in a fume hood at room temperature for 2-3 days. Washed Celite was stored in sealed brown bottles.

Residues of extracts were chromatographed on Celite using either the gradient elution technique described by Engel et al (55) or the

stepwise elution method. For both types of chromatography, the columns were prepared in the same manner. The ratio of weight of Celite to the weight of extract was between 500 and 1000 to 1. All columns had a height to diameter ratio of at least 20 to 1. Celite was mixed thoroughly with stationary phase (0.5 ml of stationary phase per g of Celite) in a plastic bag and portions of approximately 10 g of Celite were transferred quickly to a suitable glass chromatographic column and were packed with a Martin packer. After all the Celite was packed, mobile phase was allowed to flow through the column. The hold-back volume of the column was then measured by the procedure described by Johnson (56). This method involved measuring the volume of mobile phase required to elute the dye, Sudan IV, from the column. The hold-back volumes of the Celite columns used in these studies ranged from 1.5 to 2.0 ml per g of Celite. Extracts were packed as a charge on top of the column. A minimum amount of stationary phase was used to dissolve the extract and an appropriate quantity of Celite, used in the same ratio as that for packing the column, was added to this solution. The mixture was stirred to ensure thorough mixing and was then packed on top of the column and covered with sand. Mobile phase was delivered continuously from a reservoir attached to the top of the column and 5-10 ml fractions were collected into tubes held in an automatic fraction collector (Buchler Instruments, N.J.). Fractions were collected at a rate of 0.2 to 0.4 ml/g of Celite per hour. An aliquot from every second or third tube was taken for counting and fractions comprising a peak of radioactivity were pooled. In the gradient elution method, at least six hold-back volumes were collected before the gradient was applied and, in the stepwise elution method, either a single concentration of

mobile phase or increasing concentrations of t-butanol in the mobile phase was used to elute steroids from Celite columns. After most of the radioactivity was eluted from a column, methanol was used to strip the column. All chromatographic solvent systems used in these studies are shown in Table 2.

Silica Gel Column Chromatography

Urinary extracts were first chromatographed on large silica gel columns. Silica gel (100-200 mesh, Davison Chemical Co., Baltimore, Md.) was used as purchased, and the ratio of weight of silica gel to the weight of extract was between 100 and 200 to 1. Columns of silica gel were prepared by transferring a slurry of this support in the initial developing solvent to a suitable glass chromatographic column, while tapping the column vigorously to ensure even settling of the silica gel and to prevent the formation of air bubbles. All columns had a height to diameter ratio of at least 30. The extract to be chromatographed was dissolved in a minimum volume of the starting solvent and was applied on top of the column. Steroids were eluted from the column by increasing the concentration of absolute ethanol in methylene dichloride in a stepwise manner. Effluent from the columns was collected into tubes at the rate of 30-40 ml per hour. Aliquots of fractions were removed from every second or third tube for the determination of radioactivity, and fractions comprising a peak of radioactivity were pooled. All silica gel columns were stripped with methanol after most of the radioactivity was eluted from the columns.

Small silica gel columns (usually 1-2 g) were used for further purification of steroids eluted after paper chromatography. These columns were prepared in the manner described above.

Table 2

Solvent Systems Used in Chromatography

System	Type of Chromatography	Solvents
A	CPC*	Iso-octane:methanol:water (20:9:1) Gradient - iso-octane:ethylene dichloride (1:1)
B	CPC	Iso-octane:t-butanol:water:NH_4OH (12:20:19:1)
C1	CPC	Iso-octane:t-butanol:water:NH_4OH (8:20:19:1)
C2	CPC	Iso-octane:t-butanol:water:NH_4OH (6:20:19:1)
D	CPC	Benzene:cyclohexane:methanol:water (2:2:3:3)
E	CPC	Iso-octane:t-butanol:methanol:water (10:4:1:5)
F	CPC	Iso-octane:t-butanol:methanol:water (20:8:3:9)
G	PPC**	Toluene-propylene glycol
H	PPC	Benzene:cyclohexane (1:1) - propylene glycol
I	PPC	Ligroin C-propylene glycol
J	PPC	Ligroin B:methanol:water (10:9:1)
K	PPC	Ligroin C:methanol:water (4:3:1)
L	PPC	Benzene:cyclohexane:methanol:water (1:2:3:3)
M	PPC	Benzene:methanol:water (2:1:1)
N	PPC	Iso-octane:t-butanol:methanol:water (10:2:7:1)
O	PPC	Iso-octane:toluene:methanol:water (5:5:7:3)
P	PPC	Toluene:methanol:water (4:3:1)
Q	PPC	Ligroin C:benzene:methanol:water (10:5:8:2)
R	PPC	Iso-octane:toluene:methanol:water (4:4:4:1)
S	PPC	Ligroin C:toluene:methanol:water (5:15:14:6)
T	PPC	Isopropyl ether:t-butanol:1M NH_4OH:H_2O (6:4:1:9)

Table 2 (Cont'd)

System	Type of Chromatography	Solvents
U	PPC	Methylcyclohexane:ethyl acetate:n-butanol: methanol:1M NH_4OH (25:40:8:20:30)
V	PPC	Iso-octane:methanol:water (10:9:1)
W	PPC	Benzene:n-butanol:methanol:water (10:1:3:3)
X	PPC	Toluene:ethyl acetate:methanol:water (9:1:6:4)
Y	PPC	Benzene:ethyl acetate:methanol:water (3:2:3:2)
Z	TLC***	Methylene dichloride:methanol (9:1)
A1	TLC	Methylene dichloride:methanol (17:3)
A2	TLC	Ethyl acetate:cyclohexane (7:3)
A3	TLC	Benzene:ethanol (4:1)

*Celite column partition chromatography.
**Paper partition chromatography.
***Thin layer chromatography.

Alumina Column Chromatography

Alumina (200 mesh, Harshaw Chemical Co., Cleveland, Ohio) used in these studies was neutralized according to the procedure described by Solomon et al (57). After drying in the oven at 120°C and cooling, the alumina was deactivated by adding 5 ml of water per 100 g alumina and was stored in tightly stoppered containers.

Columns of alumina were prepared by slowly pouring the support into a suitable glass chromatographic column containing the developing solvent, while tapping the column. Steroids were eluted in a stepwise manner with increasing concentrations of benzene in either ligroin B or hexane, and absolute alcohol in benzene. Fractions of 1 ml per g of alumina were collected into suitable flasks and were combined on the basis of the radioactivity in each fraction or visual examination of the residue in each flask.

Paper Chromatography

Chromatography on paper was accomplished in the manner described by Bush (58) and Zaffaroni (59) using strips of Whatman No. 1 or 3 MM paper. When Zaffaroni type systems were used, papers were impregnated with acetone: propylene glycol (1:1). One commonly used Bush type system (system J) was fitted with a solvent circulating device as described by Kimball et al (60). By circulating the mobile phase for 1 hour, equilibrium within the tank was attained and the paper to be chromatographed was then placed in the tank. The mobile phase was again circulated for several minutes before being added to initiate chromatography.

Steroids were located on paper in the following manner. All radioactive compounds were detected with a Packard Model 7200 Radiochromatogram scanner. Most non-labeled steroids were situated by the method of

Kritchevsky and Kirk (61). This procedure involved dipping the chromatogram in a 10% solution of phosphomolybdic acid in ethanol and heating it for a few minutes at about 90°C. Steroids appeared as blue spots on a yellow background. Ultraviolet absorbing compounds were usually located in a Chromato-Vue cabinet (Ultraviolet Products Inc., San Gabriel, Calif.). In a few instances, the Zimmermann reagent was used to detect steroids with a ketone group, for example, 5α-pregnanedione and 5β-pregnanedione. This procedure, described by Savard (62), involved dipping the chromatogram in a 2.5N KOH solution in methanol and then in a 2% solution of dinitrobenzene in methanol. After a few minutes, steroids appeared as violet spots on a white background.

Areas of paper containing steroids to be eluted were cut into small squares and were immersed in methanol for approximately 2 hours. The eluate was filtered through a sintered glass funnel, the paper was washed four times with additional volumes of methanol and the washings were filtered. The pooled filtrate was evaporated to dryness under vacuum at 40°C. Residues obtained after eluting papers initially treated with propylene glycol were dissolved in ethyl acetate and washed twice with 1/5 volume of water to remove traces of stationary phase. The ethyl acetate was then dried over Na_2SO_4 and evaporated under vacuum at 40°C.

All solvent systems used for paper chromatography are shown in Table 2.

Thin-layer Chromatography

A number of extracts were purified on plates of silica gel. These plates were prepared by mixing silica gel G (Research Specialties Co., Richmond, Calif.) with a volume of water equivalent to twice the weight of

the silica gel and spreading the slurry on 20 x 20 cm plates to a depth of 1 mm using a Research Specialties Co. spreader. After drying in air at room temperature for approximately one hour, the plates were activated by heating them at 120°C for 30 minutes. Extracts and steroid standards were dissolved in methanol:methylene chloride (1:1) and were applied 2.5 cm from one edge of a plate. Chromatograms were then developed by the ascending method in small glass tanks with glass covers.

Steroids were located on the plates as previously described for paper chromatography, with the exception that the reagents used to locate the steroids were spotted on the plates with a Pasteur pipette. Steroids having a Δ^5-3β-hydroxyl configuration were detected by means of the Oertel reagent (63). In the presence of a steroid with a Δ^5-3β-hydroxyl function, this reagent produced a straw color which later turned pink. Areas of silica gel containing steroids to be eluted were scraped carefully from the plates into a medium porosity sintered glass funnel and acetone was then used to elute the steroids from the silica gel.

In a few instances, ready-made Eastman Chromatogram sheets (6060 Silica gel with fluorescent indicator, Fisher Scientific Co.) were used for thin-layer chromatography. These sheets proved to be very convenient because they can be scanned and immersed in acetone in the same manner as paper strips.

The solvent systems used for thin-layer chromatography are listed in Table 2.

Injection of Precursors

Precursors used in these experiments were checked for purity prior to injection. The purity of these substrates will be discussed later.

Appropriate aliquots of labeled steroids for injection were transferred to sterile counting vials using sterile pipettes. Solvents in the vials were evaporated under nitrogen.

Injections were carried out with syringes in the following manner. In Experiment 1, the residue in the counting vial was dissolved in 0.5 ml of absolute ethanol, and 5 ml of sterile isotonic saline was added. This solution was injected into the umbilical vein of a pregnant Rhesus monkey with the assistance of Dr. H. Friesen. In Experiment 4, the precursors were dissolved in 0.5 ml of absolute alcohol, and 10 ml of saline was added before injection into the antecubital vein of a pregnant subject. The syringe was washed once by drawing back blood which was then re-injected. These injections were done by Dr. I. Meeker, Department of Obstetrics and Gynecology, University of Vermont, Burlington, Vt. In Experiment 5, the substrates were dissolved in 4 ml of saline and this solution was mixed with packed, type O, Rh negative red blood cells previously cross-matched with maternal serum. The labeled steroids were mixed with 200 ml of the donor blood, 90-120 ml of which was administered into the peritoneal cavity of a human fetus in utero during a transfusion for erythroblastosis fetalis. The administration of the steroids was done by Dr. J.M. Bowman, Rh Laboratory, Winnipeg, Man.

After the administration of the precursors, unused blood, syringes, needles and vials were sent back and were then washed well with ethanol, and the radioactivity in these washes was measured. In Experiment 5, a precipitate was formed after adding ethanol to the unused blood. This precipitate was removed by centrifugation and was washed with ethanol. The extraction was repeated until there was a negligible amount of

radioactivity in the supernatant. The plastic tubing used to transfuse blood into the fetus was also washed and the washings were counted. The radioactivity adsorbed on the plastic tubing was estimated by soaking 1 cm portions of the tubing in 2 ml of methanol in counting vials for several hours and then counting the methanol extracts. In all these experiments, the total radioactivity recovered in the washes was subtracted from the radioactivity originally prepared for injection. This procedure gave the actual amount of radioactivity injected.

Urine Collection

All urine collected in these studies was kept in the frozen state until it was ready to be processed. The urine was then thawed, the volume was measured and aliquots were taken for counting and creatinine determinations.

Extraction and Defatting of Tissues

Each tissue was homogenized in a glass homogenizer or a meat grinder and was extracted four times with absolute alcohol and five times with 80% ethanol. The pooled ethanolic extracts obtained after centrifugation were dried *in vacuo* at 40°C. In order to check whether extraction was complete, an aliquot from the last supernatant was taken for counting. When a sizable amount of radioactivity was still present in the ethanol, the extraction procedure was repeated and the last extract was again checked for the presence of radioactivity. An aliquot was then taken from each tissue extract for counting.

Extracts of tissues were defatted by dissolving the residues in 70% methanol and leaving them overnight at -20°C. The precipitated lipid

was removed by centrifugation and this process was repeated twice. The pooled supernatants were dried in vacuo at 40°C.

Ether-Water Partition

In Experiment 1, unconjugated steroids were separated from conjugates by partitioning extracts in equal volumes of pre-equilibrated ether and water as follows. After transferring the extract to a separatory funnel containing ether and water, the funnel was shaken very gently and the two phases were allowed to separate. The aqueous phase (lower phase) was then transferred to a second separatory funnel and another portion of 100 ml of ether was added to this funnel before the two phases were shaken gently. The same process was repeated with the first funnel after the addition of 100 ml of water. Using the above procedure, the partition was carried out in a total of three separatory funnels. At the end of this process, the aqueous and ether phases were pooled separately, dried in vacuo at 40°C and counted.

Hydrolysis of Urinary Conjugates

Unconjugated steroids in the urine were removed by extraction with diethyl ether. Insignificant amounts of radioactivity were found in this fraction in the experiments to be reported.

A modification of the solvolytic procedure of Burstein and Lieberman (64) was employed to hydrolyze the steroid sulfates. The aqueous portion obtained after extraction of urine with diethyl ether was acidified to pH 1 with concentrated sulfuric acid. After addition of 20% (w/v) of sodium chloride, the urine was divided into five equal aliquots and each portion was extracted with a volume of tetrahydrofuran equal to

the total volume of the aqueous solution. The tetrahydrofuran was filtered through glass wool, 0.11 ml of 70% perchloric acid per 100 ml of solvent was added to the filtrate and the solution was incubated at 37°C for approximately 18 hours. The tetrahydrofuran solution was then neutralized with 0.5 ml of concentrated ammonium hydroxide per 100 ml of solution and the solvent was evaporated in vacuo at 40°C. The remaining aqueous residue was diluted with water to an appropriate volume and the hydrolyzed steroids were extracted with ethyl acetate. A volume of ethyl acetate which was twice that of the aqueous solution was divided among three separatory funnels in the ratio of 2:1:1 and the aqueous solution was passed through the funnels, using one-fifth of the solution at a time. After pooling the ethyl acetate, it was washed with 5% sodium bicarbonate until basic and with water until the washings were neutral. The ethyl acetate was then dried over Na_2SO_4, filtered and evaporated in vacuo at 40°C, giving a residue which contained the steroids excreted as sulfates.

The residual aqueous phase, as well as the alkali and water washes from the above extraction, contained the steroid glucosiduronates. Hydrolysis of these conjugates was achieved by adjusting the pH of the combined aqueous phase to 4.7 with glacial acetic acid and by adding to this solution 2% (v/v) of 2N sodium acetate buffer (pH 4.7), 500 units of β-glucuronidase (specific activity, 2000 Fishman units/mg, Baylove Chemicals, Musselburgh, Scotland) per ml and 10 ml of methylene chloride as preservative. This mixture was incubated at 37°C for five days. At the end of the incubation, a neutral extract was prepared in the same manner as described for the solvolysis. This extract contained the steroids excreted as glucosiduronates and as double conjugates with

sulfuric and glucuronic acids.

In Experiment 3, simultaneous enzymatic hydrolysis of sulfates and glucosiduronates was accomplished with the use of Glusulase (Endo Laboratories, New York), a mixture containing 73,800 units of sulfatase and 106,100 units of β-glucuronidase per ml. The hydrolysis procedure involved adjustment of urine to pH 5.2 with glacial acetic acid and addition of 5% (v/v) of 1.2N sodium acetate buffer, pH 5.2, 5 ml of Glusulase per liter of urine and 10 ml of methylene chloride. This mixture was incubated at 37°C for five days. A neutral extract was then prepared as described previously.

Determination of Radiochemical Purity of Precursors and Isolated Metabolites

The isotope dilution technique was used to establish the radiochemical purity of precursors and isolated metabolites. When a compound was found to behave as a single band of radioactivity that coincided in mobility with an authentic steroid in several chromatographic systems, an aliquot of the compound was mixed with an appropriate amount of carrier steroid and the mixture was crystallized in different solvent mixtures. A Mettler microbalance was used to weigh an aliquot of the crystals (0.5 - 1.5 mg), which was then transferred quantitatively to a vial for counting. The mother liquor from each crystallization was transferred to an accurately weighed, thin-walled Florence flask (2-3 g) and was dried under nitrogen. The flask was reweighed until a constant weight was obtained and then an aliquot of the mother liquor was taken for counting. When the specific activities of the crystals and mother liquor did not differ by more than 10%, the radiochemical purity of the compound was considered to be established. The radiochemical purity of a metabolite was confirmed by

formation of a derivative which was crystallized to constant specific activity. The total number of dpm in a metabolite was calculated from the weight of carrier added and the final specific activity of the metabolite.

Determination of Endogenous Specific Activities of Isolated Steroids

The urinary steroids isolated in Experiment 3 did not have sufficient weight to determine their specific activities by crystallization. The specific activities of these steroids were determined by acetylation with labeled acetic anhydride using the double isotope derivative principles described by Kliman and Peterson (65). The isolated compounds containing ^{14}C were acetylated with one of the standardized solutions of ^{3}H-acetic anhydride and the products were purified by paper chromatography, prior to being mixed with an appropriate amount of the carrier steroid. The mixture was then filtered through a small silica gel column and crystallized until the $^{3}H/^{14}C$ ratios of the crystals and mother liquor were constant. The specific activity of the isolated steroid was calculated according to the following expression:-

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

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

a = specific activity of DOCA (dpm ^{3}H/mg),

b = molecular weight of DOCA,

n = number of acetylatable groups,

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

c = molecular weight of the metabolite.

Standardization of ^{3}H-Acetic Anhydride

Two batches of ^{3}H-acetic anhydride (specific activity 10 μc/μM) were used in these studies. They were purchased as 20% (v/v) solutions of ^{3}H-acetic anhydride in benzene and were used directly. The specific activity of the acetic anhydride in each solution was determined by acetylation of pure crystalline deoxycorticosterone and by measurement of the specific activity of deoxycorticosterone acetate. Constant specific activity of each acetate was achieved as shown in Table 3.

Table 3

Standardization of ^{3}H-Acetic Anhydride

		Specific Activity (dpm/μg DOCA)	
	Crystallization	Crystals	Mother Liquor
Batch No. 1	1	18,400	19,800
	2	18,200	17,900
Batch No. 2	1	31,420	31,790
	2	31,270	32,320
	3	31,070	32,100

Determination of Weights of Isolated Urinary Steroids

The isolation of minute amounts of urinary 15α-hydroxylated steroids in these studies was greatly facilitated by the addition of a small amount of the labeled steroid of known specific activity into urine pools. Following hydrolysis of the urinary conjugates and purification of the extract by various chromatographic procedures, the purified fraction

containing the radioactivity was acetylated with ^{3}H-acetic anhydride and the product was mixed with the appropriate carrier steroid. The mixture was then chromatographed on a small alumina column and was crystallized until the ^{3}H to ^{14}C ratios of the crystals and mother liquor were constant. After calculating the specific activity of the isolated steroid as previously described, the weight of this steroid originally present in the urine was calculated according to the well known isotope dilution equation:-

$$A = \left(\frac{sa_1}{sa_2} - 1\right) W$$

where A = weight of the isolated steroid,

SA_1 = specific activity of the steroid added in urine,

SA_2 = specific activity of the isolated steroid, and

W = weight of the steroid added in urine.

Determination of the Percent Conversion of Injected Steroids to Urinary Metabolites

The percent conversion of an injected steroid to its urinary metabolites was calculated from the final specific activity of the metabolite and the weight of the carrier added.

Melting Point Determination

Melting points were taken on a Bock-Monoscop VI/S/Th micromelting point apparatus and were corrected, using a standard curve based on the melting points of a series of primary standards (Arthur H. Thomas Co., Philadelphia, Pa.).

NMR and Mass Spectroscopy

Nuclear Magnetic Resonance spectroscopy was done by Dr. Lois Durham, Department of Chemistry, Stanford University, California, on a Varian HR-100 spectrophotometer using deuteriochloroform ($CDCl_3$) as the solvent and tetramethylsilane as an internal standard. Observed chemical shifts were compared with shifts calculated for likely structures from the additive substituent effects in Zürcher's Tables (66).

Mass spectroscopy was performed by Dr. O. Mamer, Royal Victoria Hospital, on a LKB Model 9000 spectrometer. Mass spectra were obtained by using an ionizing current of 60 μa and an ionizing voltage of 70 ev. The ion source temperature was at 290°C and the direct inlet probe temperature was in the range of 50-100°C.

Elemental Analysis

Elemental analyses were done by Dr. G. Schilling, Ayerst Laboratories, Montreal.

Preparation of Derivatives

Acetylation

Steroids to be acetylated were desiccated for about one hour, dissolved in two parts of pyridine and one part of acetic anhydride in a stoppered tube and left in the dark at room temperature for approximately 18 hours. Anhydrous conditions were used throughout.

Acetylation was stopped by either one of two methods. The more common procedure involved the addition of benzene:methanol (1:1) to the solution and evaporation of the solvents under nitrogen at 40°C. This procedure was repeated until the odor of pyridine had disappeared. In the second method,

the reaction mixture was transferred to ice water containing 5% of 6N sulfuric acid and the acetylated product was extracted three times with ethyl acetate. The pooled ethyl acetate was then washed five times with 6N sulfuric acid, three times with 1N sodium hydroxide and with water until neutral, and was dried over Na_2SO_4, filtered and evaporated *in vacuo* at 40°C. The latter method was used when steroids were acetylated with labeled acetic anhydride.

Sodium Borohydride Reduction

Reduction of ketones with sodium borohydride was achieved by using the method of Norymberski and Woods (67). The steroid to be reduced was dissolved in methanol to give a 0.4% solution which was then cooled in ice water at 4°C. A 1.6 molar excess of $NaBH_4$ was added to the cooled solution and the reaction was allowed to proceed for one hour at 4°C. At the end of this period, the reaction was stopped by the addition of glacial acetic acid and the methanol was evaporated. After dissolving the residue in water, the reduced steroid was extracted with ethyl acetate. This extract was washed with 5% sodium bicarbonate solution and then with water until neutral. It was dried over Na_2SO_4 and filtered before being evaporated *in vacuo* at 40°C.

Dichlorodicyanobenzoquinone Oxidation

The preparation of some derivatives involved oxidation with DDQ, a reagent which selectively oxidizes allylic alcohols. This reaction was carried out in a stoppered test tube using the procedure of Burn et al (68). The steroid to be oxidized was dissolved in freshly distilled dioxane (1.5% solution) to which was added a 1.25M excess of DDQ; the reaction was

allowed to proceed in the dark for 24-30 hours. The hydroquinone formed was removed by filtration, the yellow filtrate was evaporated to dryness and the residue obtained was dissolved in ethyl acetate. This solution was washed with 0.5N NaOH and then with water until neutral, before it was dried over Na_2SO_4, filtered and the organic solvent was evaporated *in vacuo* at 40°C.

Preparation and Purification of Carrier Steroids

15α-Hydroxyandrostenedione

15α-Hydroxyandrostendione was prepared by the microbiological hydroxylation of androstenedione with a strain of Penicillium (ATCC 11598) as described by Stern et al (42). Starting with 440 mg of androstenedione, a crude extract weighing 826 mg was obtained at the end of incubation. This extract was purified on a 100 gm alumina column. Elution with 1% ethanol in benzene gave a residue weighing 351 mg. Crystallization of this material from a mixture of methanol and hexane yielded 146 mg of crystals, mp 196-197°C. The infrared spectrum (KBr) of this compound was identical to that of authentic 15α-hydroxyandrostenedione.

15α-Hydroxyestradiol

Impure 15α-hydroxyestradiol (obtained from Ayerst Laboratories) was purified by silica gel column chromatography. Approximately 500 mg of the impure material was applied on a 60 gm silica gel column and 15α-hydroxyestradiol was eluted with 8% ethanol in methylene dichloride, giving a residue which weighed 84 mg. Crystallization from a mixture of methanol and hexane yielded 63 mg of crystals, mp 248-249°C. The infrared spectrum (KBr) of this material was identical to that of authentic 15α-hydroxyestradiol.

15α-Hydroxydehydroisoandrosterone, 15α-Hydroxydehydroisoandrosterone diacetate, Androst-5-ene-3β,15α,17β-triol triacetate

The preparation and purification of these three new compounds will be described later in this thesis.

Preparation and Purification of Labeled Precursors

4-^{14}C-Pregnenolone

When purchased, 4-^{14}C-pregnenolone had a specific activity of 56 mc/mM. An aliquot of this material was shown to be radiochemically pure by the isotope dilution technique, as shown in Table 4. The purity of this compound was calculated to be over 99%.

4-^{14}C-15α-Hydroxyandrostenedione

4-^{14}C-15α-Hydroxyandrostenedione was synthesized by the microbiological hydroxylation of 4-^{14}C-androstenedione (specific activity 58.8 mc/mM) as described by Stern et al (42). The latter compound was first checked for purity by chromatographing an aliquot on paper in system K (8 hours) where it migrated as a single band of radioactivity. A total of 3.40×10^8 dpm of the 4-^{14}C-androstenedione was diluted with 2.88 mg of crystalline androstenedione and the mixture was incubated according to the procedure indicated above. After the incubation, extraction of the hydroxylated steroid gave a residue which weighed 61 mg and contained 3.14×10^8 dpm. This material was chromatographed sequentially on paper in systems G (18 hours), L (31 hours) and M (3 hours). In the last chromatogram, a single symmetrical peak of radioactivity, which had the same mobility as authentic 15α-hydroxyandrostenedione, was detected. Elution of this material yielded a residue which contained 1.65×10^8 dpm.

It was shown to be radiochemically pure after isotope dilution was carried out on an aliquot of this compound as shown in Table 5. The purity of this material was calculated to be 95%.

7-^{3}H-15α-Hydroxyandrostenedione

Approximately 2.0 x 10^{8} dpm of impure 7-^{3}H-15α-hydroxyandrostenedione (specific activity 1.66 x 10^{6} dpm/μg) was obtained from Mr. Herman Schut of our laboratory. This material was purified by chromatography on paper in systems M (4 hours), N (64 hours), O (24 hours), P (4 hours) and M (4 hours). The last chromatogram showed a single band of radioactivity and this material was shown to be radiochemically pure (Table 6). The purity of this compound was over 97%.

4-^{14}C-15α-Hydroxydehydroisoandrosterone

The preparation and purification of this compound will be described later in this thesis.

Table 4

Proof of Radiochemical Purity of 4-^{14}C-Pregnenolone

	Specific Activity (dpm/mg)	
Crystallizations	Crystals	Mother Liquor
1	2240	2250
2	2220	2290
Calculated	2250*	

*A total of 40,340 dpm of 4-^{14}C-pregnenolone was mixed with 17.90 mg of carrier pregnenolone prior to crystallization.

Table 5

Proof of Radiochemical Purity of 4-^{14}C-15α-Hydroxyandrostenedione

	Specific Activity (dpm/mg)	
Crystallizations	Crystals	Mother Liquor
1	1760	2060
2	1730	1980
3	1740	1810
Calculated	1830*	

*A total of 72,740 dpm of 4-^{14}C-15α-hydroxyandrostenedione was mixed with 39.75 mg of carrier 15α-hydroxyandrostenedione prior to crystallization.

Table 6

Proof of Radiochemical Purity of 7-^{3}H-15α-Hydroxyandrostenedione

	Specific Activity (dpm/mg)	
Crystallizations	Crystals	Mother Liquor
1	3860	4120
2	3800	4100
3	3820	3820
Calculated	3840*	

*A total of 151,250 dpm of 7-^{3}H-15α-hydroxyandrostenedione was combined with 39.40 mg of carrier 15α-hydroxyandrostenedione prior to crystallization.

EXPERIMENTAL SECTION AND RESULTS

Experiment 1 - Metabolism of Pregnenolone by the Fetal Tissues and Placenta of the Rhesus Monkey

A pregnant Rhesus monkey (Macaca mulatta) was used in this experiment. She was anesthetized with nembutal and the uterus was exposed through a mid-abdominal incision. After a small opening was made in the uterine wall and the amniotic sac, the umbilical cord was located and a portion of it was exposed. A solution containing 2.41×10^7 dpm of ^{14}C-pregnenolone was injected into the umbilical vein over a period of 1 minute, 20 seconds and the cord was left intact for another 2 minutes, 10 seconds before it was clamped and the fetus was delivered. A few minutes later the placenta was separated and removed from the uterine wall. The fetus was female and had a crown-rump length of 11.0 cm and weighed 110 g. From these observations (69) and the equation of Payne and Wheeler (70), the age of the fetus was estimated to be 95-100 days. Various fetal tissues and the placenta were dissected, weighed, placed in absolute ethanol and stored in the freezer. The weights of these tissues are shown in Table 7.

The experimental design used in the processing of the tissues is shown in Figure 1. Each tissue was homogenized, defatted and subjected to an ether-water partition as described earlier. The total radioactivity in each tissue and the distribution of this radioactivity between ether and water are shown in Table 7.

Experimental Design

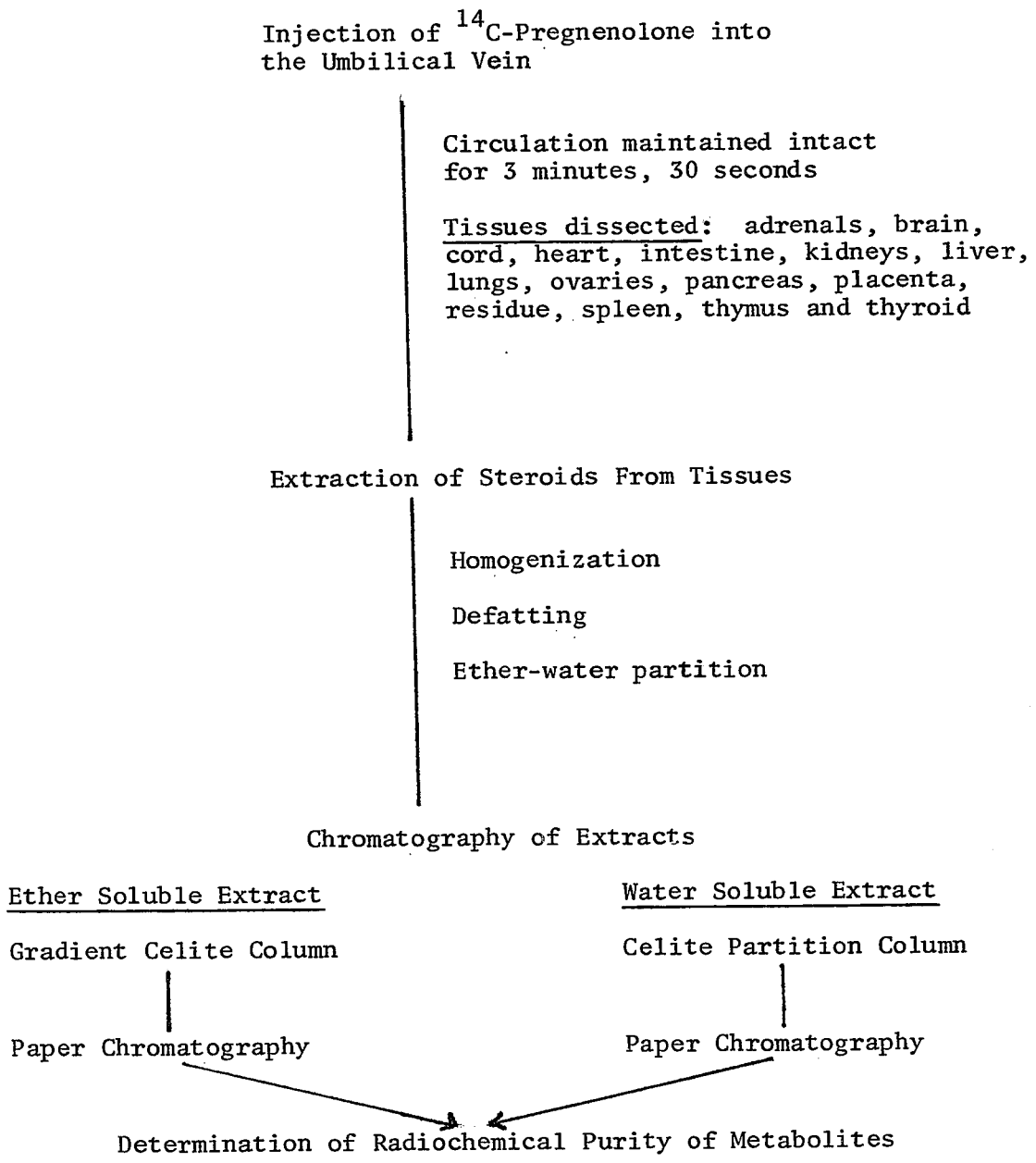


Fig. 1. Experimental design used to extract, purify and establish radiochemical purity of metabolites isolated from the placenta and fetal tissues of the Rhesus monkey.

Table 7

Weight and Radioactivity of Fetal Tissues and Placenta of the Rhesus Monkey After the Injection of ^{14}C-Pregnenolone

Tissue	Weight (g)	Total Radioactivity in Tissue (dpm x 10^5)	Percent of Total dpm in an Ether Soluble Form
Adrenals	0.05	0.46	99
Brain	11.70	21.30	99
Cord	1.20	7.16	100
Heart	0.70	11.24	99
Intestine	2.55	7.26	98
Kidneys	0.60	4.01	99
Liver	3.60	19.95	84
Lungs	1.20	6.75	99
Ovaries	*	0.01	96
Pancreas	0.20	7.56	99
Placenta	25.00	3.81	100
Residue	85.65	116.52	92
Spleen	0.20	3.68	99
Thymus	0.15	0.18	98
Thyroid	0.05	0.24	98
		210.13	

*Negligible

Adrenals

The ether extract of the adrenals contained 45,850 dpm and weighed 5.5 mg; it was purified directly by paper chromatography. A scheme of its purification is shown in Figure 2. The extract was first chromatographed in system J for 7 1/2 hours and it separated into four zones of radioactive material. They were designated A to D in increasing order of polarity. (This order of polarity will be the same in the other purification schemes shown in this section of the thesis.) Residues A, C and D were not processed, since they contained very little radioactivity. The residue from the major zone, B, was chromatographed in system H for 11 hours and only a single peak of radioactivity was detected on the chromatogram. In both systems zone B had the same mobility as pregnenolone, run as a standard. The radioactive material eluted from the last chromatogram contained 33,600 dpm. Half of this material was mixed with 34.30 mg of carrier pregnenolone and the mixture was crystallized to constant specific activity as shown in Table 8.

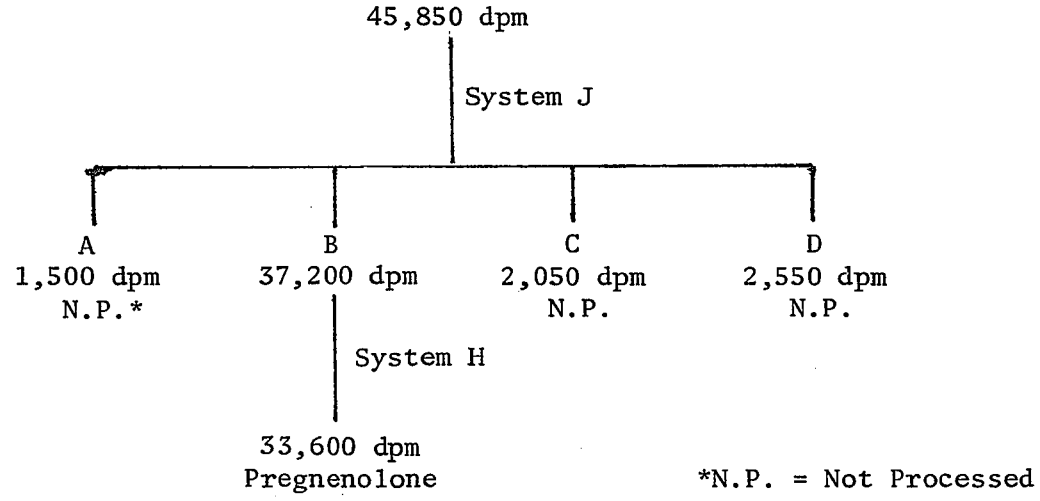


Fig. 2. Purification of the ether extract of the adrenals.

Table 8

Proof of Radiochemical Purity of Pregnenolone Isolated From the Ether Extract of the Adrenals

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	410	690
2	420	410
3	400	380
Calculated	480*	

*A total of 16,350 dpm was mixed with 34.30 mg of carrier pregnenolone prior to crystallization.

Brain

The ether extract of the brain weighed 142.5 mg and contained 2,113,340 dpm. It was chromatographed on a 130 gm Celite column using system A and 10 ml fractions were collected. All the radioactivity on the column was recovered without applying the gradient. The graph obtained by plotting cpm per fraction against fraction number is shown in Figure 3, and the weight and radioactivity of the pooled fractions from the column are given in Table 9.

Since the residues from Pools I, II and IV contained small amounts of radioactivity, only the material of Pool III was processed further. This material, which contained 1,971,500 dpm, was chromatographed on paper in system J for 3 1/4 hours, and three zones of radioactivity were detected on the chromatogram as shown in Figure 4. The material from the major zone, IIIB, was further chromatographed in system N for 11 1/4 hours.

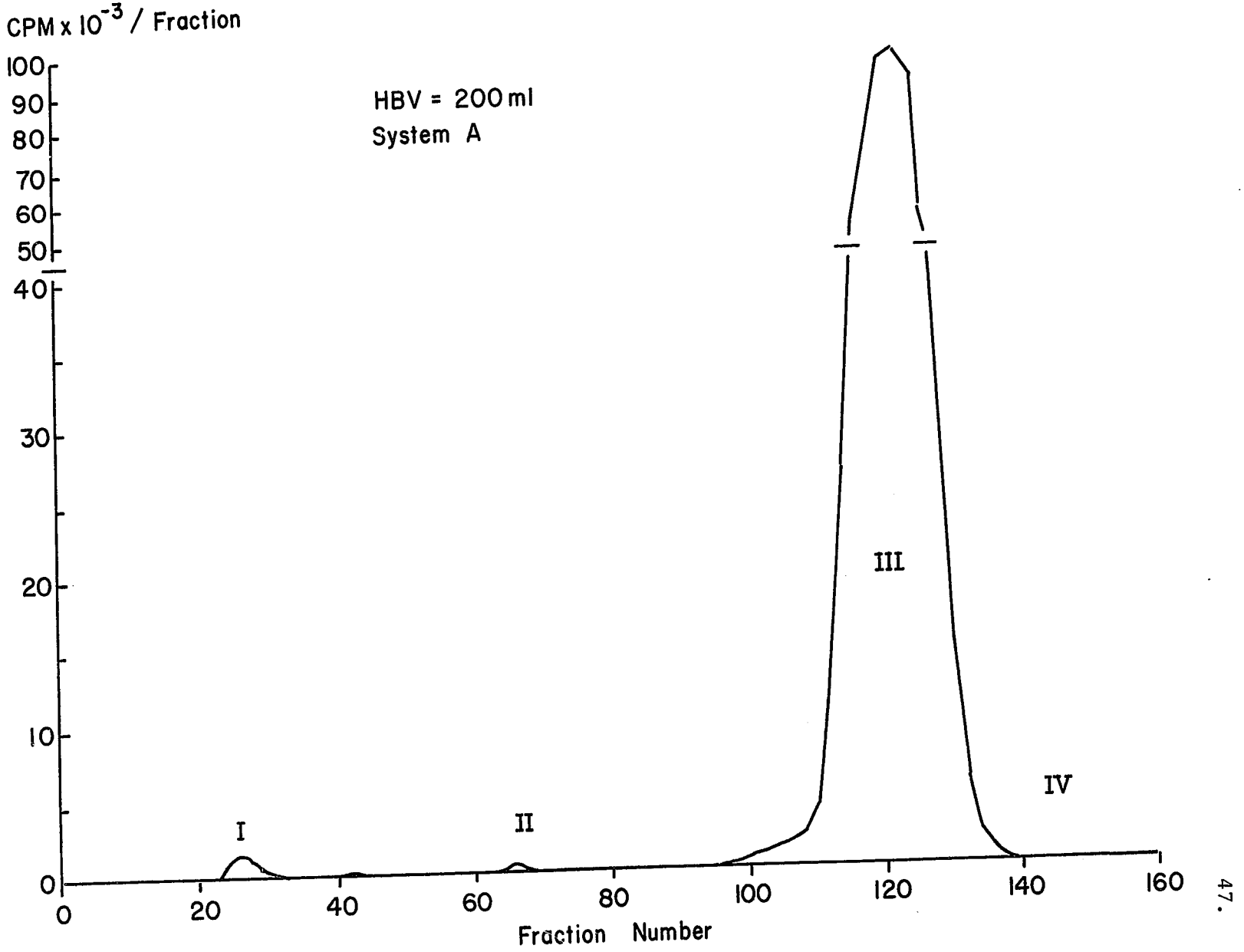
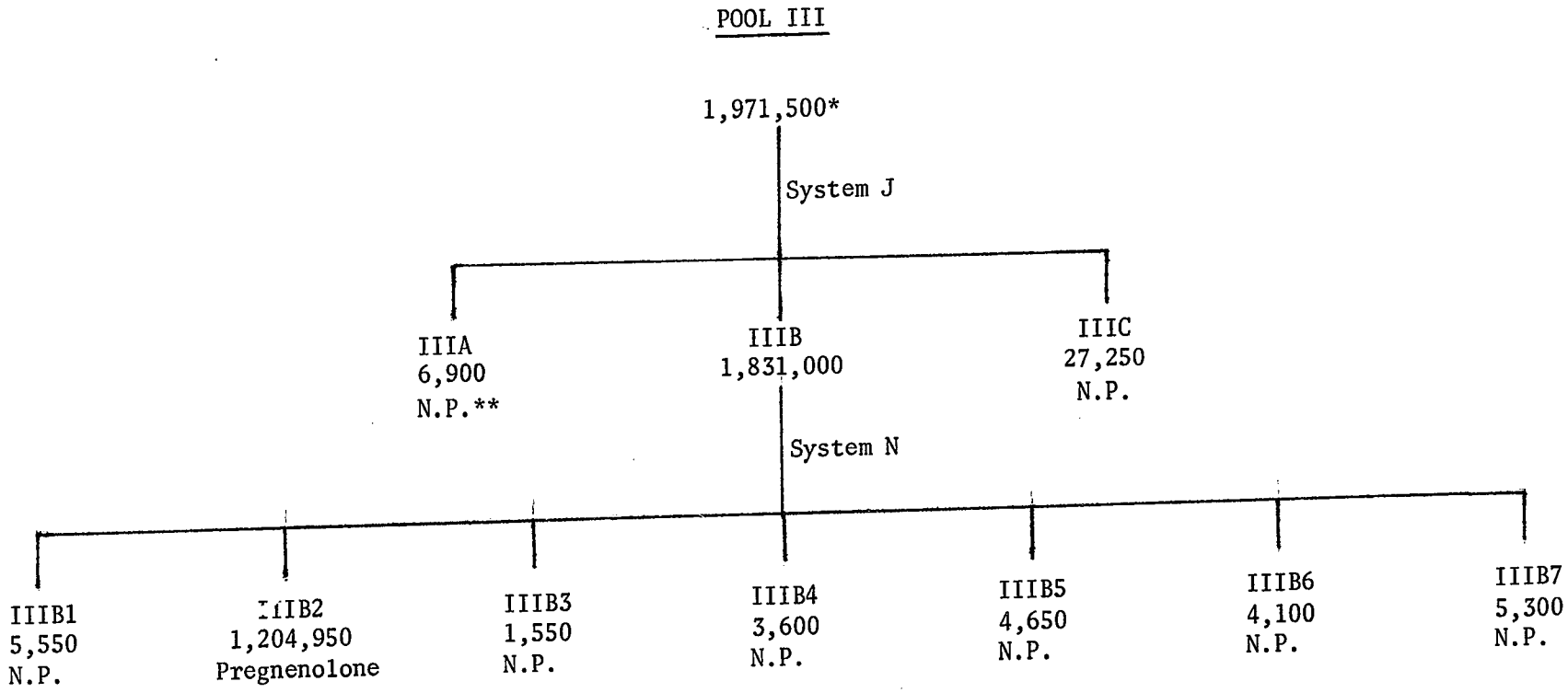


Fig. 3. Celite column chromatography of ether extract of brain.



*Numbers are in dpm
**N.P. = Not Processed

Fig. 4. Purification of the material in Pool III obtained after Celite column chromatography of the ether extract of the brain.

In both systems, the major radioactive zone had the same mobility as pregnenolone. An aliquot of the eluate from zone IIIB2 was mixed with 45.85 mg of carrier pregnenolone and the mixture was crystallized to constant specific activity as shown in Table 10. Zone IIIC did not migrate off the origin and, on elution, yielded a very oily residue which was not purified further. Also, the remaining residues shown in Figure 4 contained an insufficient amount of radioactivity for further purification.

Table 9

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Ether Extract of the Brain

Pool No.	Fractions Pooled	Weight (mg)	dpm
I	24-32	51.1	12,400
II	33-97	9.9	9,680
III	98-138	2.4	1,971,500
IV	139-160	0.4	5,400
Strippings		48.0	45,320

Table 10

Proof of Radiochemical Purity of Pregnenolone Isolated From the Ether Extract of the Brain

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	4770	2570
2	4670	5740
3	4680	4980
4	4670	4800
Calculated	3690*	

*A total of 169,400 dpm was mixed with 45.85 mg of carrier pregnenolone prior to crystallization.

Cord

The ether extract of the cord had a weight of 4.3 mg and contained 715,900 dpm; it was purified directly by paper chromatography in system J for 3 1/4 hours. Two zones of radioactive material were detected on the chromatogram. The minor radioactive zone did not migrate from the origin and, on elution, yielded 10,400 dpm of oily material which was not processed further. The material from the major zone, which gave 600,800 dpm on elution, was further chromatographed in system N for 11 3/4 hours and a single peak of radioactivity was detected on the chromatogram. The eluted radioactive material, which had the same mobility as authentic pregnenolone in both systems, contained 598,000 dpm. An aliquot of this material was mixed with 24.70 mg of carrier pregnenolone and the mixture was crystallized to constant specific activity as shown in Table 11.

Table 11

Proof of Radiochemical Purity of Pregnenolone

Isolated From the Ether Extract of the Cord

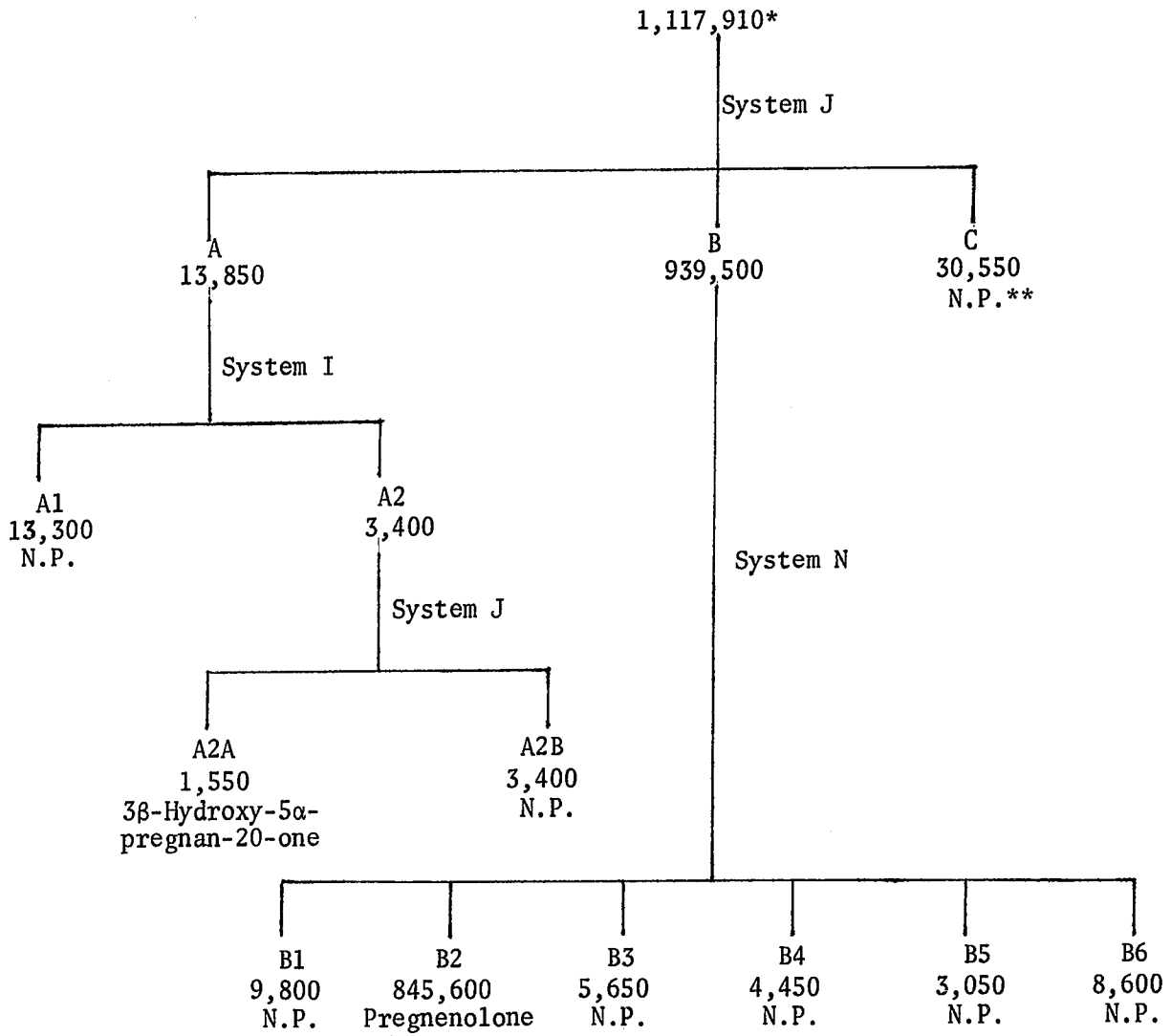
	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	7280	7500
2	6930	7040
3	6840	7140
Calculated	6760*	

*A total of 167,000 dpm was mixed with 24.70 mg of carrier pregnenolone prior to crystallization.

Heart

The ether extract of the heart contained 1,117,910 dpm and weighed 7.5 mg and it was purified by paper chromatography in system J for 3 1/2 hours as shown in the scheme in Figure 5. Only the residues from zones A and B were processed. Zone C remained at the origin and, on elution, yielded a very oily residue. Residue A was further chromatographed in system I for 4 hours and resolved into two peaks of radioactive material. Zone A2, which had the same mobility as standard 3β-hydroxy-5α-pregnan-20-one, was rechromatographed in system J for 5 hours and two radioactive zones were detected. The less polar zone, A2A, which had the same mobility as 3β-hydroxy-5α-pregnan-20-one, yielded 1,550 dpm on elution. This material was mixed with 10.30 mg of carrier 3β-hydroxy-5α-pregnan-20-one and was shown to be radiochemically pure (Table 12). A total of 3.5 mg of the last crystals was acetylated and the product formed was crystallized to constant specific activity (Table 12). The infrared spectrum (KBr) of this material was identical to that of authentic 3β-acetoxy-5α-pregnan-20-one.

The major radioactive material, B, which migrated with the same mobility as pregnenolone in system J (3 1/2 hours) and system N (10 1/2 hours), yielded 845,600 dpm on elution from the last chromatogram. An aliquot of this eluate was mixed with 40.35 mg of pregnenolone and the mixture was crystallized to constant specific activity as shown in Table 13. The remaining radioactive materials shown in Figure 5 either contained an insufficient amount of radioactivity for further processing or did not migrate with the same mobility as any of the steroids run as standards.



*Numbers are in dpm
**N.P. = Not Processed

Fig. 5. Purification of the ether extract of the heart.

Table 12

Proof of Radiochemical Purity of 3β-Hydroxy-5α-pregnan-20-one Isolated From the Ether Extract of the Heart

	Specific Activity (dpm/mg)			
Crystallization	3β-Hydroxy-5α-pregnan-20-one Crystals	3β-Hydroxy-5α-pregnan-20-one Mother Liquor	3β-Acetoxy-5α-pregnan-20-one Crystals	3β-Acetoxy-5α-pregnan-20-one Mother Liquor
1	97	109	79	105
2	104	97	81	83
3	97	105		
Calculated	97*		86**	

*A total of 1,550 dpm was mixed with 10.30 mg of carrier 3β-hydroxy-5α-pregnan-20-one prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 3β-hydroxy-5α-pregnan-20-one and adjusting for the change in molecular weight.

Table 13

Proof of Radiochemical Purity of Pregnenolone Isolated From the Ether Extract of the Heart

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	6580	7090
2	6490	6270
3	6390	6360
Calculated	6760*	

*A total of 272,700 dpm was mixed with 40.35 mg of carrier pregnenolone prior to crystallization.

Intestine

The ether extract of the intestine was purified by paper chromatography as shown in Figure 6. This extract, which weighed 26.0 mg and contained 716,740 dpm, was first chromatographed in system J for 3 1/2 hours and it resolved into three zones of radioactivity. Zone C did not migrate off the origin and was not processed further. Zone A was further chromatographed in system I for 4 hours and two radioactive bands were detected on the chromatogram. In this system, zone A1 was less polar than 5α-pregnanedione, run as a standard, and was not processed further, whereas the material in the more polar area, A2, was rechromatographed in system J for 6 1/4 hours. The latter zone, which had the same mobility as 3β-hydroxy-5α-pregnan-20-one, yielded 760 dpm on elution and the eluate was mixed with 10.00 mg of carrier 3β-hydroxy-5α-pregnan-20-one. This mixture was crystallized to constant specific activity as shown in Table 14. The final crystals and mother liquor had a combined weight of 3.7 mg and were acetylated. The resulting product was then crystallized to constant specific activity as shown in Table 14. The infrared spectrur (KBr) of this material matched that of authentic 3β-acetoxy-5α-pregnan-20-one.

Residue B (Figure 6) was further purified in system N for 12 hours and, as in the previous system, it had the same mobility as standard pregnenolone. Elution of the radioactive material in zone B2 gave 501,800 dpm. An aliquot of this material was mixed with 40.45 mg of carrier pregnenolone and the mixture was crystallized to constant specific activity as shown in Table 15. Residue B1 was not processed further since its mobility did not coincide with that of any of the steroids run as standards.

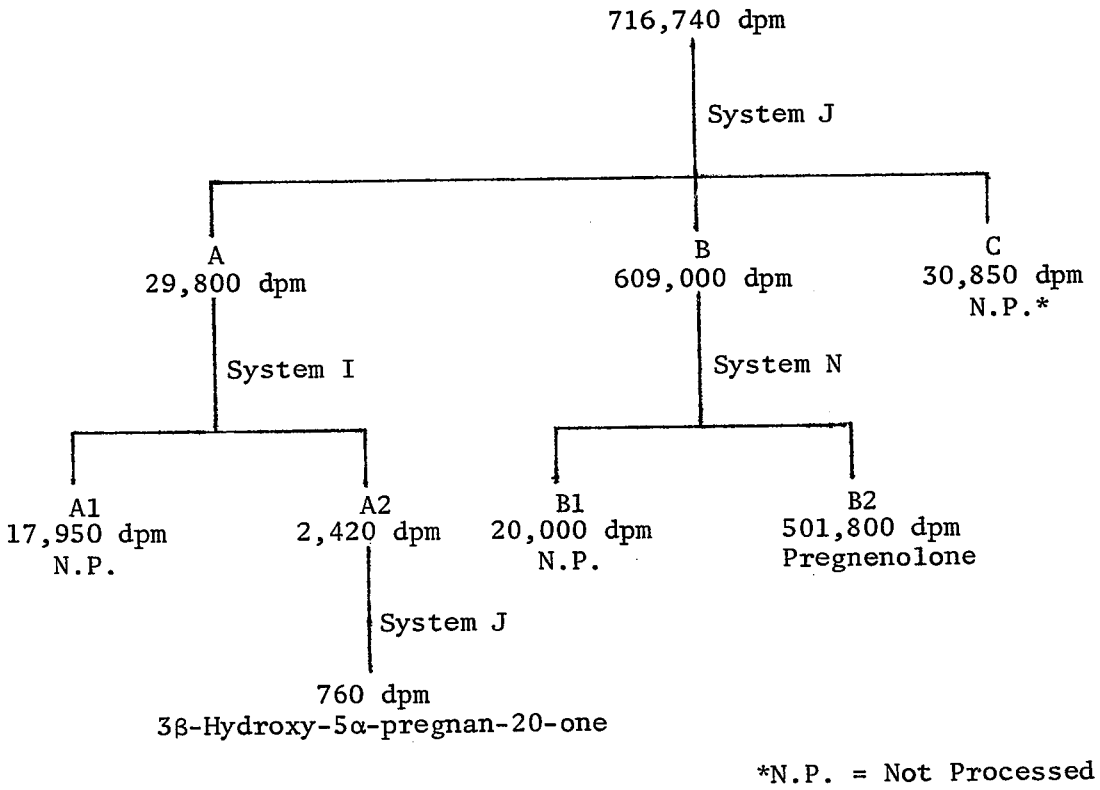


Fig. 6. Purification of the ether extract of the intestine.

Table 14

Proof of Radiochemical Purity of 3β-Hydroxy-5α-pregnan-20-one Isolated From the Ether Extract of the Intestine

	Specific Activity (dpm/mg)			
Crystallization	3β-Hydroxy-5α-pregnan-20-one Crystals	3β-Hydroxy-5α-pregnan-20-one Mother Liquor	3β-Acetoxy-5α-pregnan-20-one Crystals	3β-Acetoxy-5α-pregnan-20-one Mother Liquor
1	69	88	60	129
2	67	68	63	68
3	72	75		
Calculated	76*		66**	

*A total of 760 dpm was mixed with 10.00 mg of carrier 3β-hydroxy-5α-pregnan-20-one prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 3β-hydroxy-5α-pregnan-20-one and adjusting for the change in molecular weight.

Table 15

Proof of Radiochemical Purity of Pregnenolone Isolated From the Ether Extract of the Intestine

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	3870	4420
2	3700	4480
3	3760	3580
Calculated	5100*	

*A total of 206,200 dpm was mixed with 40.45 mg of carrier pregnenolone prior to crystallization.

Kidneys

The ether extract of the kidneys weighed 8.9 mg and contained 397,490 dpm, and it was chromatographed on paper as outlined in Figure 7. Three radioactive bands were detected on the chromatogram after purifying the extract in system J for 4 hours. The residue from the least polar zone, A, was rechromatographed in system J for 3 hours and it separated into two bands of radioactivity, one of which migrated close to 5α-pregnanedione, run as a standard. However, after further chromatography of residue A1 in system I for 4 hours, it was obvious that the mobility of this material was much different from that of 5α-pregnanedione and the material could not be identified. Residue B was purified further in system N for 11 1/2 hours and was found to migrate with the same mobility as standard pregnenolone. Elution of the radioactive material in the last chromatogram gave 251,400 dpm. An aliquot of this eluate was mixed with 68.00 mg of carrier pregnenolone and the mixture was crystallized to constant specific activity as shown in Table 16.

Table 16

Proof of Radiochemical Purity of Pregnenolone
Isolated From the Ether Extract of the Kidneys

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	1410	1680
2	1420	1420
3	1380	1430
Calculated	1440*	

*A total of 97,800 dpm was mixed with 68.00 mg of carrier pregnenolone prior to crystallization.

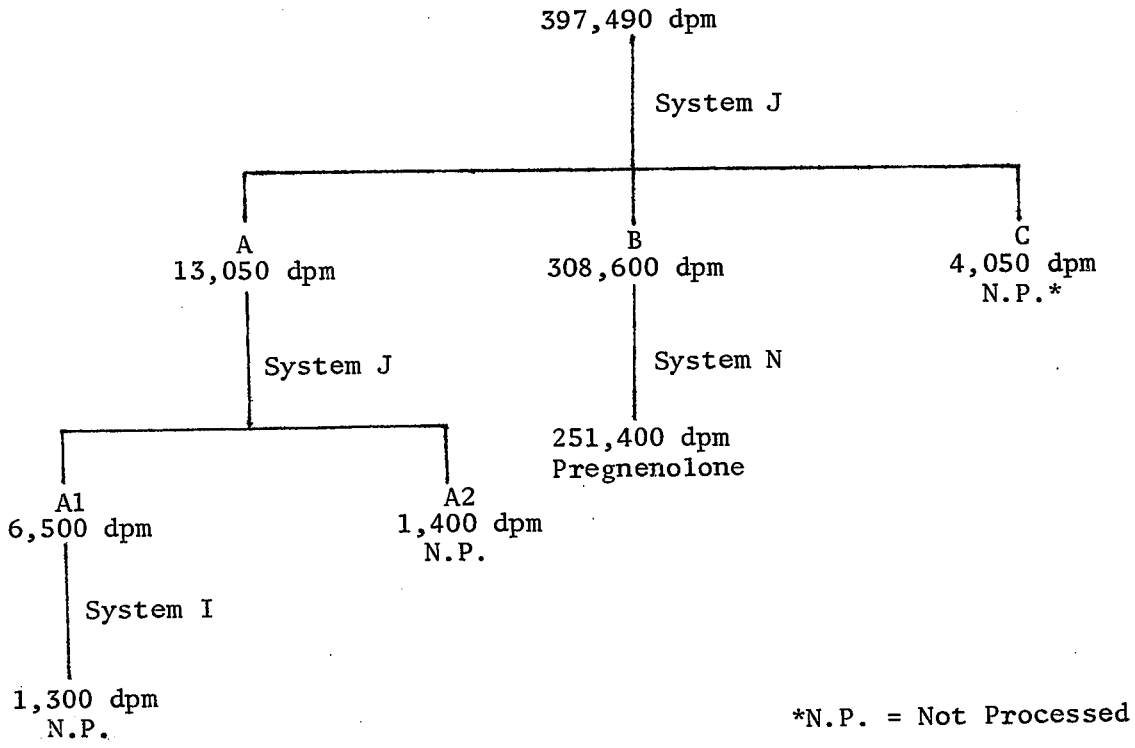


Fig. 7. Purification of the ether extract of the kidneys.

Lungs

The ether extract of the lungs weighed 9.7 mg and contained 670,200 dpm; it was purified directly by paper chromatography and the purification scheme is shown in Figure 8. The extract was resolved into three peaks of radioactive material after being chromatographed in system J for 3 1/2 hours. Residue A was further purified in system I for 4 hours and two bands of radioactivity were detected on the chromatogram. Zone A1 was much less polar than 5α-pregnanedione and was not processed further. Zone A2 separated into three bands of radioactivity and the residues from these areas contained an insufficient amount of radioactivity for further processing. Residue B was further purified in system N for 11 hours and a single peak of radioactive material was detected on scanning the chromatogram. In both systems, zone B had the same mobility as pregnenolone. Elution of the radioactive material in the last chromatogram yielded 459,700 dpm. An aliquot of this eluate was mixed with 43.30 mg of carrier pregnenolone prior to crystallization and the mixture was crystallized to constant specific activity as shown in Table 17. The residue from zone C, which contained 16,450 dpm and which did not migrate off the origin, was not processed further.

Table 17

Proof of Radiochemical Purity of Pregnenolone Isolated From the Ether Extract of the Lungs

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	4580	4950
2	4440	4670
3	4480	4430
Calculated	4680*	

*A total of 202,700 dpm was mixed with 43.30 mg of carrier pregnenolone prior to crystallization.

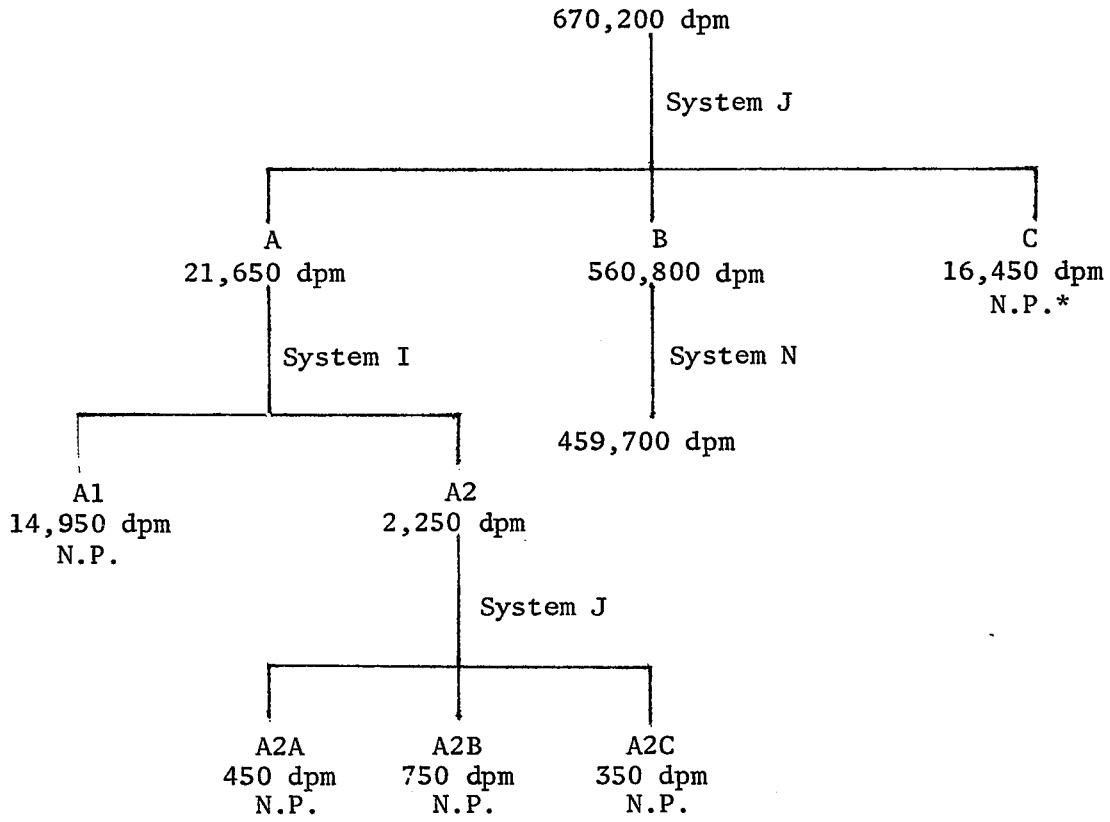


Fig. 8. Purification of the ether extract of the lungs.

Liver

The ether extract of the liver weighed 44.9 mg and contained 1,691,040 dpm. It was chromatographed on a 35 gm Celite column in system A and 10 ml fractions were collected. Gradient elution was started after twenty hold-back volumes had been collected. The cpm per fraction were plotted against fraction number as shown in Figure 9, and the radioactivity of the pooled fractions is shown in Table 18. The residues from Pools IV, VIII, X, XI and XIII were not processed further.

Table 18

Weight and Radioactivity of Pooled Fractions Obtained After Gradient Celite Column Chromatography of the Ether Extract of the Liver

Pool No.	Fractions Pooled	Weight (mg)	dpm
I	9-35	26.4	43,350
II	36-51	3.4	34,100
III	52-73	5.2	598,000
IV	74-177	12.8	42,500
V	178-191	1.4	10,280
VI	192-215	3.7	9,880
VII	216-259	13.3	52,050
VIII	260-304	4.2	43,650
IX	305-327	2.4	287,200
X	328-344	1.3	44,750
XI	345-355	1.0	15,400
XII	356-365	0.8	187,000
XIII	366-375	1.8	45,300
XIV	376-437	3.8	9,500
Strippings		3.4	100,560

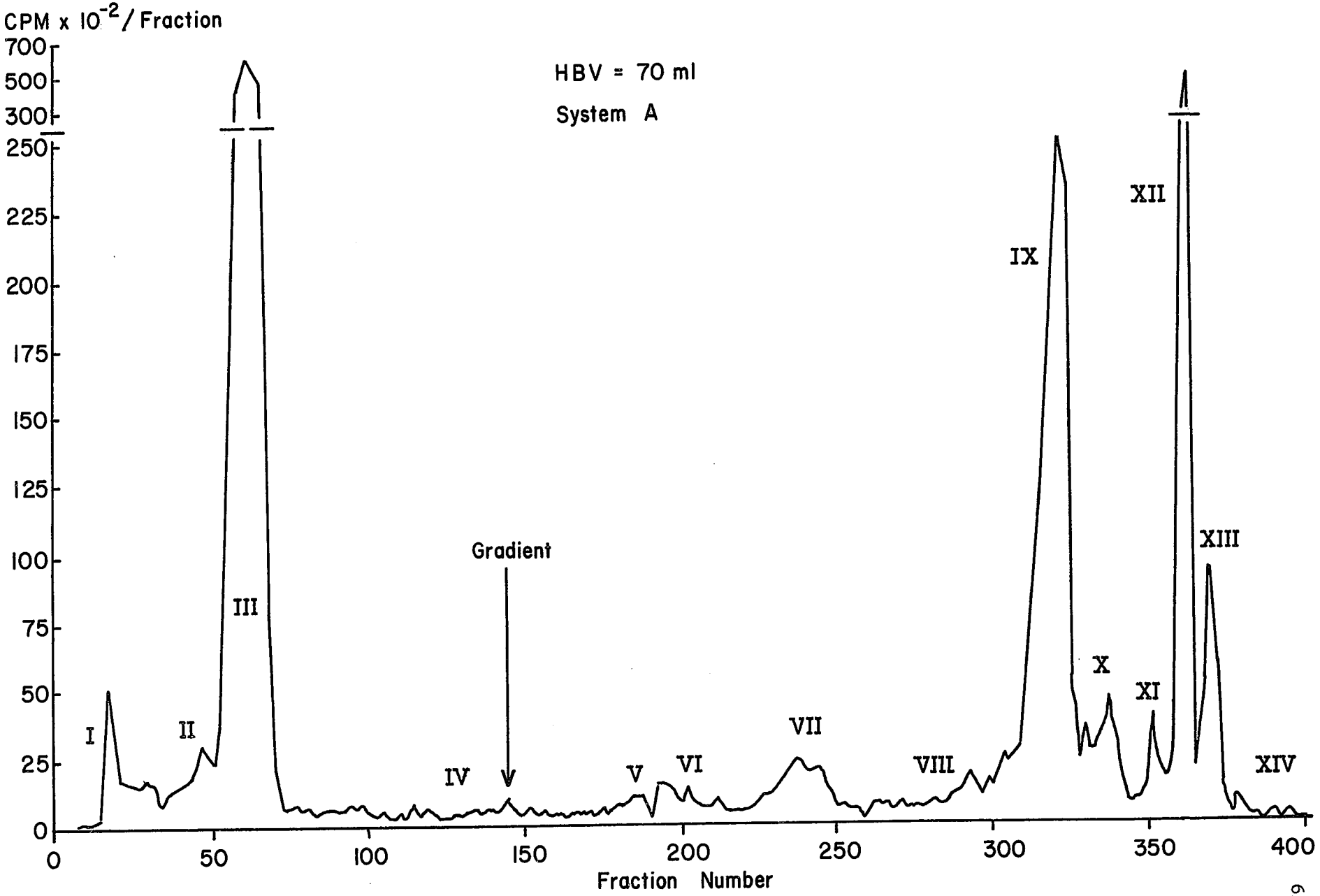


Fig. 9. Gradient Celite column chromatography of ether extract of liver.

The material in Pool I contained 43,350 dpm and was chromatographed on paper in system I for 4 3/4 hours, yielding six zones of radioactive material, and the scheme for purifying these zones is outlined in Figure 10. The materials from zones B, C, D, E and F were not further analyzed because each zone appeared as a broad band of radioactivity on scanning the chromatogram and yielded a small amount of radioactivity on elution. Zone A migrated as 5α-pregnanedione and the residue from this zone contained 14,050 dpm. Half of this material was mixed with 31.65 mg of carrier 5α-pregnanedione and the mixture was crystallized. After two crystallizations, all the counts partitioned into the mother liquors. The remaining portion of residue A was mixed with 30.20 mg of carrier 5β-pregnanedione and again all the counts partitioned into the mother liquors after two crystallizations. Thus, residue A remained unidentified.

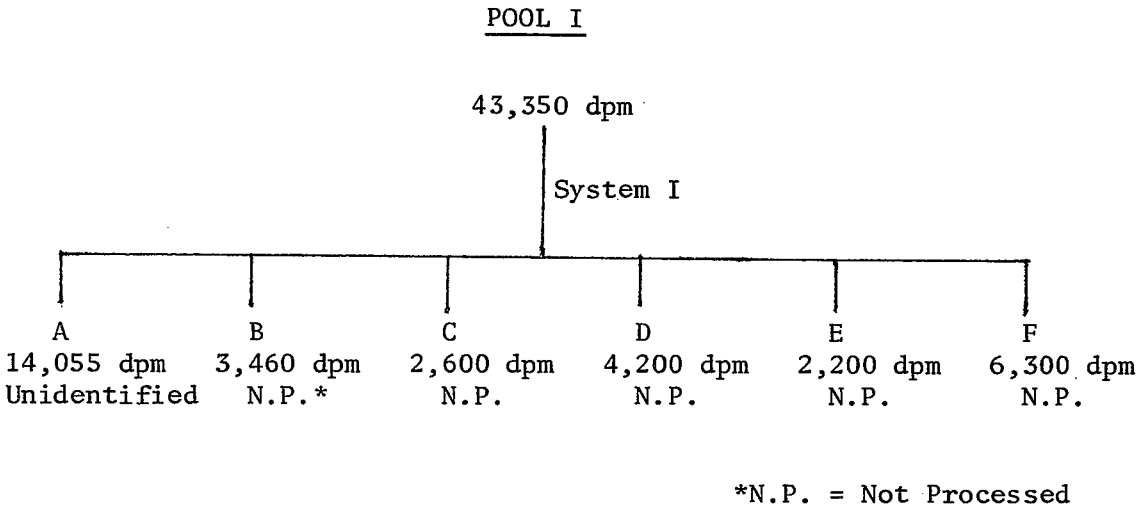


Fig. 10. Purification of the material in Pool I obtained after Celite column chromatography of the ether extract of the liver.

The residue from Pool II (34,100 dpm) was purified by paper chromatography as shown in Figure 11. It was chromatographed first in system J for 3 1/2 hours giving five radioactive bands. Only zone C was further purified, since the other areas contained small amounts of radioactivity. Residue C was chromatographed in system I for 7 hours and it resolved into three bands of radioactivity. The material from zone C1, which migrated as progesterone, was then purified sequentially in system K for 5 hours and system N for 3 hours. In the last system, two peaks of radioactive material were detected on scanning the chromatogram. Residue C1A1 was rechromatographed in system J for 3 3/4 hours and a single peak of radioactive material, which had the same mobility as progesterone, was obtained. Elution of this zone yielded 2,050 dpm which were combined with carrier progesterone and crystallized to constant specific activity as shown in Table 19. A total of 10.2 mg of the last crystals was reduced with $NaBH_4$ and then oxidized with DDQ as described earlier. The oxidized product was chromatographed on a 2 g alumina column, and 9.1 mg of residue containing 800 dpm was eluted with benzene. This material was then crystallized to constant specific activity (Table 19) and the infrared spectrum (KBr) of the last crystals was identical to that of authentic 20β-dihydroprogesterone.

The material in zone C1A2 was rechromatographed in system J for 5 1/2 hours and migrated as a single peak of radioactive material with the same mobility as pregnanolone. The eluate from this chromatogram contained 2,850 dpm and was mixed with 14.35 mg of carrier pregnanolone and the mixture was crystallized. After two crystallizations, all of the counts went into the mother liquors and the material in zone C1A2 remained unidentified.

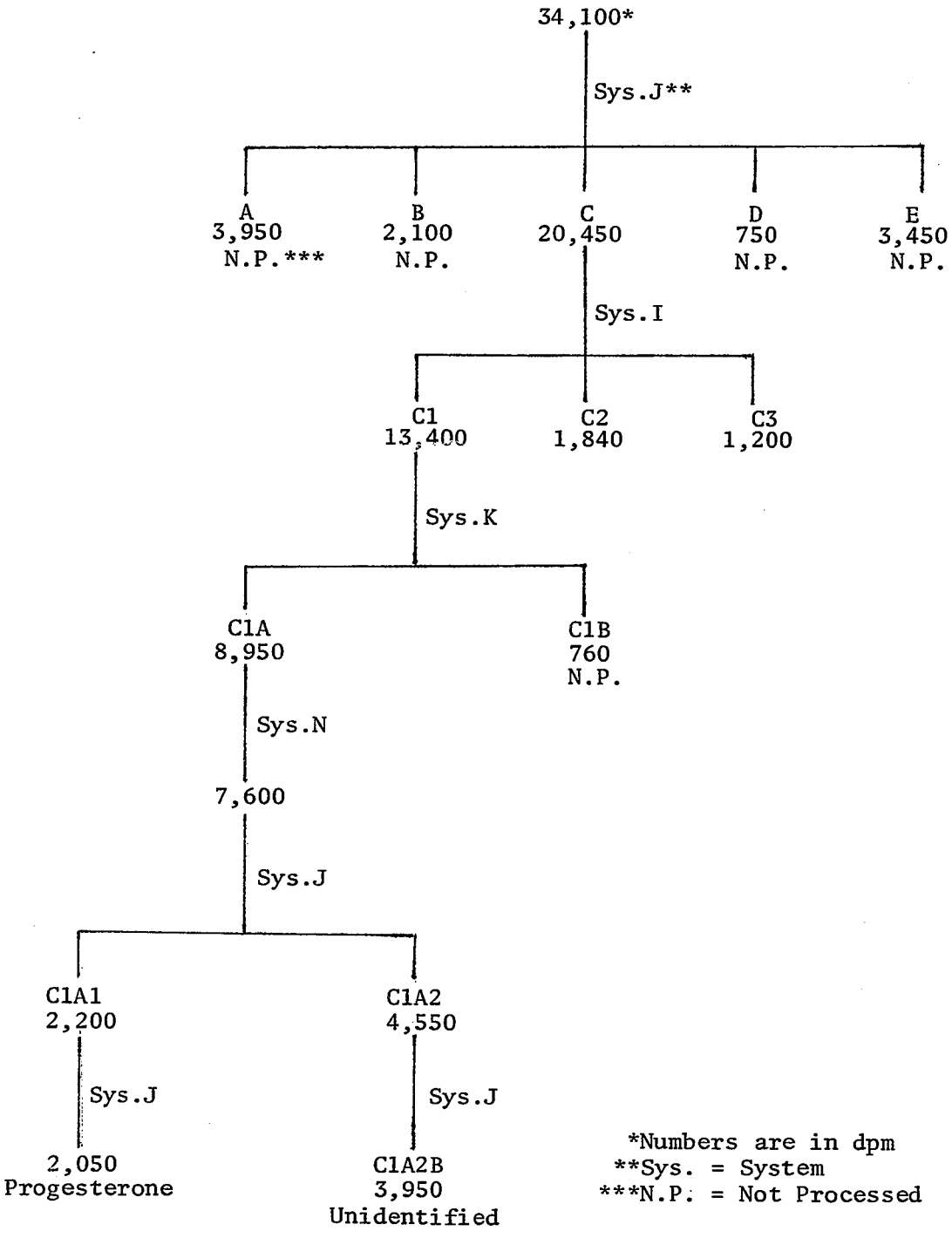


Fig. 11. Purification of the material in Pool II obtained after Celite column chromatography of the ether extract of the liver.

Table 19

Proof of Radiochemical Purity of Progesterone

Isolated From the Ether Extract of the Liver

	Specific Activity (dpm/mg)			
Crystal-lization	Progesterone Crystals	Mother Liquor	20β-Dihydroprogesterone Crystals	Mother Liquor
1	84	94	81	53
2	87	86	81	84
Calculated	103*		86**	

*A total of 2,050 dpm were mixed with 19.90 mg of carrier progesterone prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of progesterone and adjusting for the change in molecular weight.

The residue from Pool III contained 598,000 dpm and was purified by chromatography on paper as outlined in the scheme in Figure 12. The material was first chromatographed in system H for 7 1/2 hours, yielding two peaks of radioactivity. The residue from the major zone, A, was chromatographed in system J for 5 hours; however, the chromatography was poor and a sizable amount of radioactivity was lost. Two areas were eluted from the chromatogram. Zone A1 migrated with the same mobility as authentic 3β-hydroxy-5α-pregnan-20-one and the residue obtained after elution of the radioactive material was rechromatographed in system J for 5 1/4 hours. The material eluted from the chromatogram contained 42,100 dpm and half of this material was mixed with 30.05 mg of carrier 3β-hydroxy-5α-pregnan-20-one. After three crystallizations, the residue from zone A1 was found to be radiochemically pure as shown in Table 20. The remainder of the third

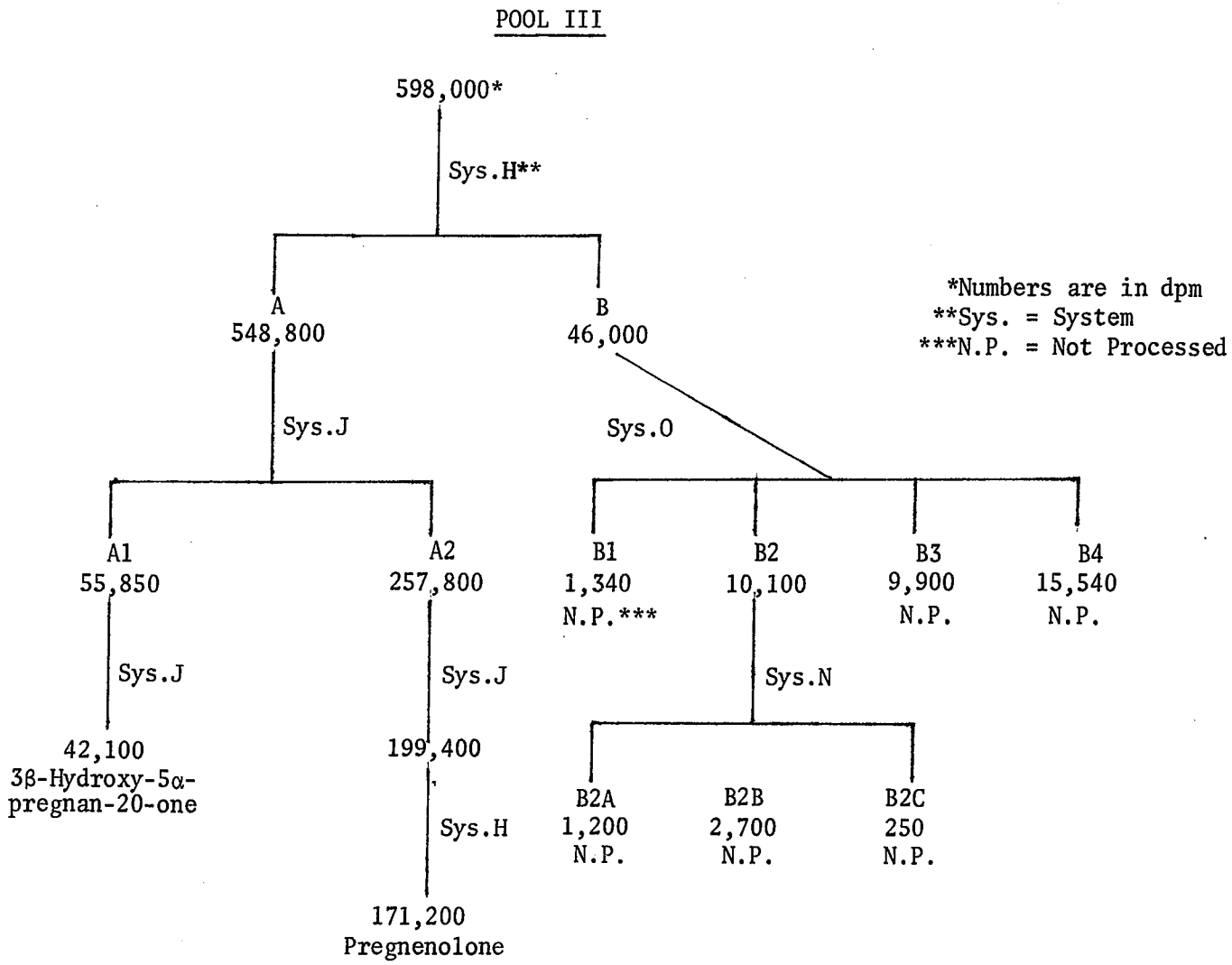


Fig. 12. Purification of the material in Pool III obtained after Celite column chromatography of the ether extract of the liver.

crystals (12.2 mg) was acetylated and the acetylated product was crystallized to constant specific activity as shown in Table 20. Infrared analysis (KBr) of an aliquot of the last crystals gave a spectrum which was identical with that of authentic 3β-acetoxy-5α-pregnan-20-one.

The material from zone A2, which migrated as pregnenolone, was chromatographed sequentially in system J for 5 hours and system H for 8 hours and migrated as a single peak of radioactive material in both systems. The eluate from the last chromatogram contained 166,700 dpm and half of this material was mixed with carrier pregnenolone. Constant specific activity was achieved after three crystallizations of the mixture as shown in Table 21.

Residue B was chromatographed in system O for 3 hours, yielding four very broad bands of radioactivity (zones B1-B4). Only the material from zone B2 was processed further. After chromatography of this residue in system 5 for 18 3/4 hours, three radioactive areas were detected on the chromatogram but were not processed further, since they contained very little radioactivity.

The residue from Pool V contained 10,280 dpm and was chromatographed in system O for 3 1/2 hours. On scanning the chromatogram, a peak of radioactive material, which had the same mobility as standard pregn-5-ene-3β,20α-diol, was detected and the eluate from this radioactive zone contained 5,940 dpm. However, further chromatography of this residue in system J for 25 1/2 hours revealed that the unknown was neither pregn-5-en-3β,20α-diol nor the 20β-epimer. An insufficient amount of radioactivity was left for further processing.

Table 20

Proof of Radiochemical Purity of 3β-Hydroxy-5α-pregnan-20-one Isolated From the Ether Extract of the Liver

	Specific Activity (dpm/mg)			
Crystallization	3β-Hydroxy-5α-pregnan-20-one Crystals	3β-Hydroxy-5α-pregnan-20-one Mother Liquor	3β-Acetoxy-5α-pregnan-20-one Crystals	3β-Acetoxy-5α-pregnan-20-one Mother Liquor
1	680	640	530	950
2	690	640	590	700
3	700	680	580	600
Calculated	710*		620**	

*A total of 21,400 dpm was mixed with 10.05 mg of carrier 3β-hydroxy-5α-pregnan-20-one prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 3β-hydroxy-5α-pregnan-20-one and adjusting for the change in molecular weight.

Table 21

Proof of Radiochemical Purity of Pregnenolone Isolated From the Ether Extract of the Liver

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	1500	1540
2	1510	1550
3	1560	1520
Calculated	1700*	

*A total of 84,200 dpm was mixed with 49.80 mg of carrier pregnenolone prior to crystallization.

The material in Pool VI (9,880 dpm) was purified by paper chromatography in system G for 5 hours and the major radioactive band on the chromatogram migrated close to pregn-5-ene-3β,20α-diol, run as a standard. Further chromatography of the eluted material in system Q for 8 hours and system N for 8 hours showed that this material had the same mobility as pregnanediol. Elution of the radioactive material from the last chromatogram yielded 1,750 dpm which were then mixed with 14.85 mg of carrier pregnanediol. After a single crystallization, an insignificant amount of radioactivity remained in the crystals and nothing further was done with the unknown compound.

The residue from Pool VII, containing 52,050 dpm, was chromatographed on paper in system R for 4 hours and resolved into four broad peaks of radioactivity (Figure 13). Only zone B migrated close to any of the steroids run as standards. Subsequently, the residue from this zone was chromatographed sequentially in system J for 8 1/2 hours, system N for 8 hours and system Q for 8 1/2 hours. In the last two chromatograms, a single zone of radioactive material was detected and it had the same mobility as 21-hydroxypregnenolone, run as a standard. Elution of the radioactive material from the last chromatogram afforded 6,150 dpm. Since 21-hydroxypregnenolone is very difficult to crystallize, half of the eluted material was acetylated and the product was mixed with 26.30 mg of carrier 21-hydroxypregnenolone diacetate and the mixture was chromatographed on a 3 g alumina column. Elution with 40% benzene in ethanol gave a residue weighing 23.8 mg and containing 4,300 dpm. Crystallization of this material resulted in the partitioning of all the radioactivity into the mother liquor. Nothing further was done with this unknown compound.

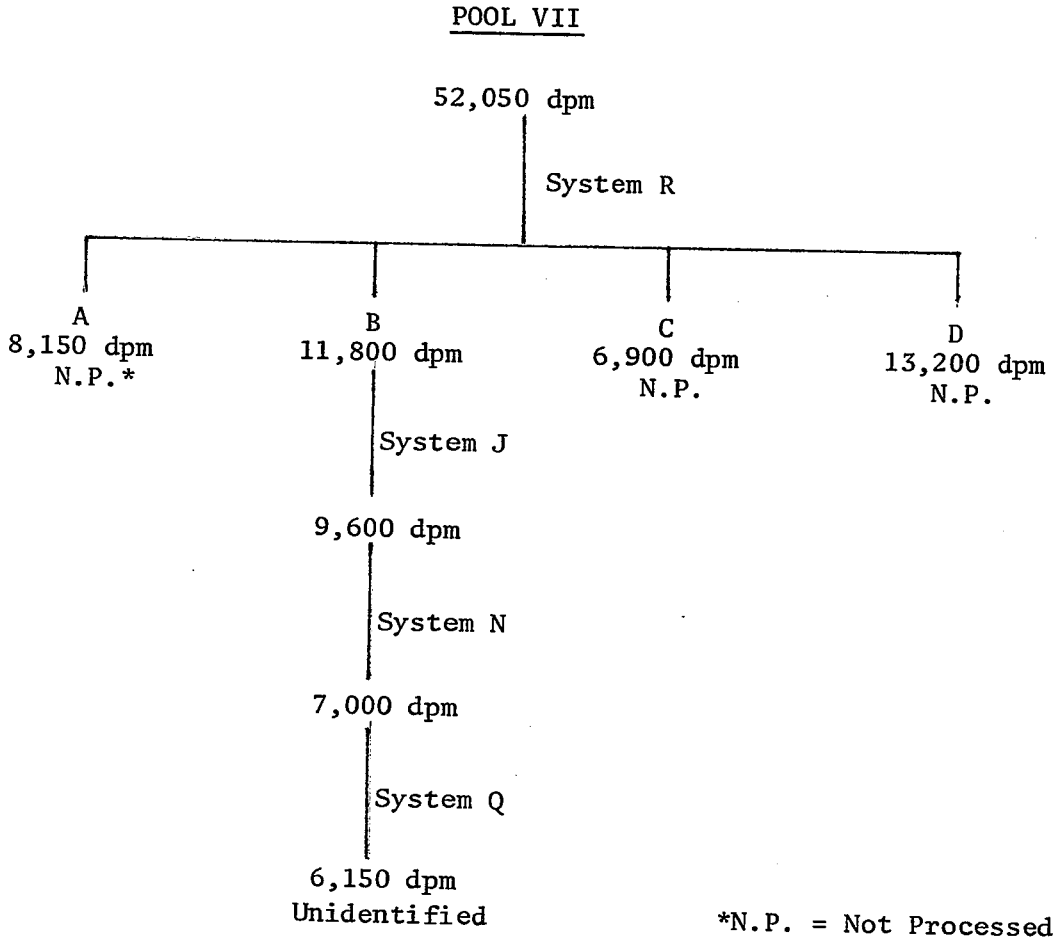


Fig. 13. Purification of the material in Pool VII obtained after Celite column chromatography of the ether extract of the liver.

The material in Pool IX (287,000 dpm) was chromatographed in system O for 4 hours and in system S for 3 1/2 hours. In each system, a single symmetrical peak of radioactive material, which had the same mobility as 17α-hydroxypregnenolone, was detected on scanning the chromatogram. Elution of the material from the radioactive zone in the last chromatogram yielded 220,000 dpm. Since 17α-hydroxypregnenolone is difficult to crystallize, one-third of the eluted material was acetylated and the product was mixed with 50.10 mg of carrier 17α-hydroxypregnenolone-3-acetate. This mixture was chromatographed on a 5 g silica gel column

and elution with 3% ethanol in methylene dichloride yielded a residue weighing 47.6 mg and containing 55,400 dpm. After three crystallizations of this residue, all the radioactivity was found in the mother liquors. The remaining portion of the residue obtained from the last chromatogram was purified further in system G for 40 hours, however, the peaks of radioactive material detected did not coincide in mobility with either androst-5-ene-3β,17β-diol or 3β,17α-dihydroxy-5α-pregnan-20-one, run as standards. Nothing further was done with the materials eluted from the radioactive zones.

The material in Pool XII contained 187,000 dpm and was purified by paper chromatography. It was first chromatographed in system M for 11 hours and separated into four radioactive zones. Three of the zones contained less than 3,000 dpm and were not processed further. The residue from the major area of radioactivity was rechromatographed in system M for 4 1/2 hours and was then purified in system N for 30 hours. In both systems a single band of radioactivity migrated as 16α-hydroxypregnenolone, run as a standard. Elution of the radioactive material in the last chromatogram gave 102,700 dpm. An aliquot of this material was mixed with 40.25 mg of carrier 16α-hydroxypregnenolone and, after a single crystallization of the mixture, an insignificant amount of radioactivity remained in the crystals. The remaining portion of the material from the radioactive zone in the last chromatogram was purified sequentially in system G for 60 hours and system Q for 80 hours, and in each system a single peak of radioactivity with the same mobility as 16α-hydroxydehydroisoandrosterone, run as a standard, was detected. The eluted material from the last chromatogram afforded 38,800 dpm and half of this residue was mixed with 35.30 mg of

carrier 16α-hydroxydehydroisoandrosterone. After two crystallizations of this mixture, all the radioactivity partitioned into the mother liquors.

The aqueous extract of the liver weighed 84.3 mg and contained 304,250 dpm. It was purified by Celite column chromatography in system B and 10 ml fractions were collected. The solvent system was changed to system C1 after seven hold-back volumes and again after the sixteenth hold-back volume to system C2. The cpm per fraction were plotted against fraction number as shown in Figure 14, and the weight and radioactivity of the pooled fractions are shown in Table 22. The materials from Pools I - IV were processed further.

Table 22

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Aqueous Extract of the Liver

Pool No.	Fractions Pooled	Weight (mg)	dpm
I	9-26	10.6	96,500
II	27-69	6.0	21,950
III	70-83	2.8	12,100
IV	84-96	2.6	26,750
V	97-138	5.1	33,500
VI	139-154	5.1	19,500
VII	155-228	19.7	7,250
Strippings		51.0	60,200

The residue from Pool I (96,500 dpm), which was eluted in the second, third and fourth hold-back volumes, was purified by paper

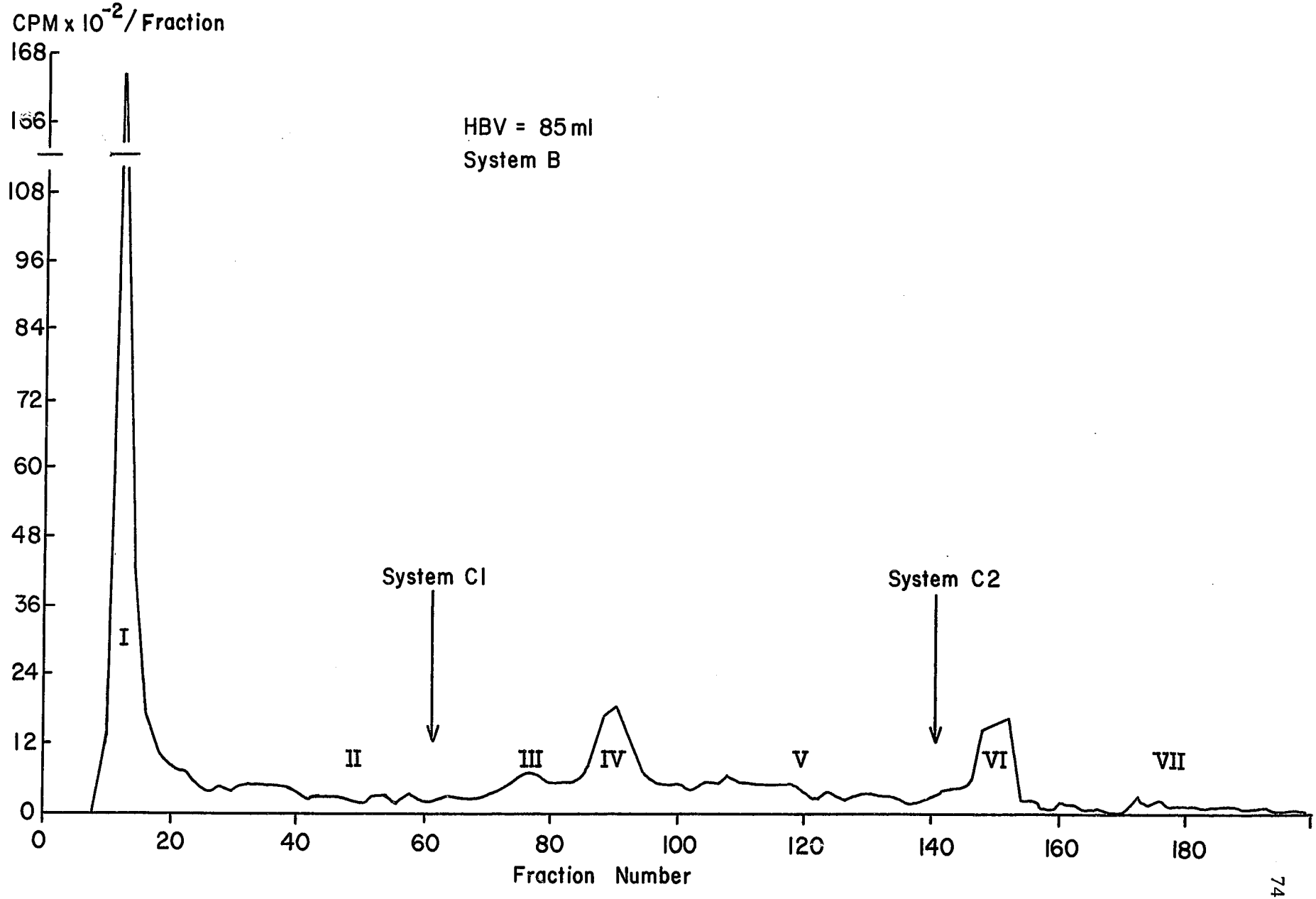


Fig. 14. Celite column chromatography of aqueous extract of liver.

chromatography in system M for 4 hours, yielding four broad zones of radioactive material (Figure 15). Only the materials from zones A and D were processed further. Residue A was further chromatographed in system J for 4 1/2 hours and separated into three bands of radioactivity. The material in zone A1 was purified in system I for 6 1/2 hours and the single radioactive area detected on the chromatogram migrated between pregnenolone and progesterone, run as standards. Elution of the radioactive zone gave 560 dpm which could not be processed further. Residue A3 was chromatographed in system J for 3 hours, however, the band of radioactivity remained at the origin of the chromatogram and nothing further was done with the eluate from this zone. Residue D from the first chromatogram was chromatographed in system T for 4 hours and resolved into two zones of radioactivity, neither of which migrated as any of the steroids run as standards. The materials from these zones were not purified further.

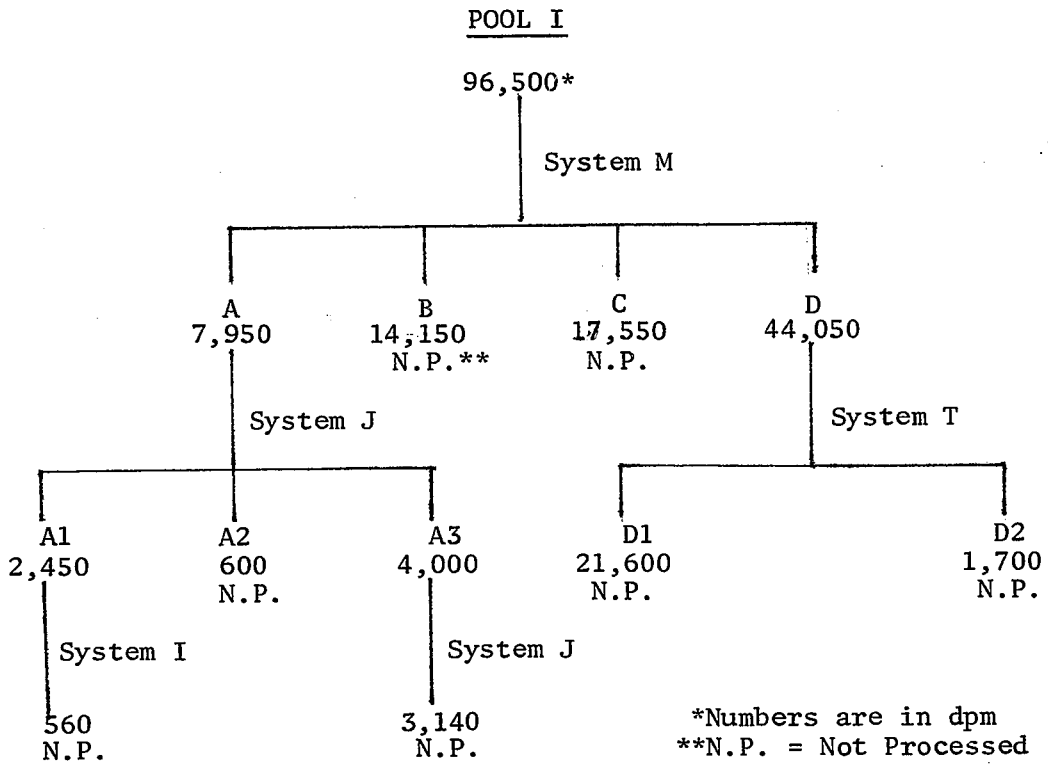


Fig. 15. Purification of the material in Pool I obtained after Celite column chromatography of the aqueous extract of the liver.

The material in Pool II contained 21,950 dpm and was chromatographed in system T for 7 1/4 hours, yielding six zones of radioactive material. Since each zone contained less than 5,000 dpm, nothing further was done with these areas.

The residue from Pool III (12,100 dpm) was purified by paper chromatography in system T for 28 hours and the single zone of radioactivity detected on the chromatogram had the same mobility as deoxycorticosterone sulfate, run as a standard. However, after further chromatography of the material (9,750 dpm) from this zone in system U, a broad band of radioactivity was detected, and it was obvious from the mobility of this unknown compound that it was not deoxycorticosterone sulfate. Nothing further was done with the resulting eluate which contained 3,900 dpm.

The residue in Pool IV contained 26,750 dpm and was chromatographed sequentially in system T for 18 3/4 hours and in system 13 for 28 hours. In both systems, a single peak of radioactive material migrated with the same mobility as authentic 16α-hydroxypregnenolone-3-sulfate. Since this steroid was not available in sufficient amounts required for use as carrier, the material eluted from the radioactive area in the last chromatogram was solvolyzed according to the procedure described by Bhavnani and Solomon (71) and the product, which contained 8,000 dpm, was purified by paper chromatography in system M for 4 hours. On scanning, a single radioactive area was detected at the origin of the chromatogram, whereas 16α-hydroxypregnenolone, run as a standard, migrated approximately two-thirds of the length of the paper from the origin. The residue (5,700 dpm) from the eluted zone was then subjected to an enzymatic

hydrolysis with β-glucuronidase as described earlier. The product of the hydrolysis, which contained 4,950 dpm, was chromatographed in system M for 4 hours and two radioactive bands were detected. The less polar zone had the same mobility as 16α-hydroxypregnenolone, run as a standard, but yielded only 220 dpm on elution; the more polar zone was found at the origin of the paper and gave 3,000 dpm. Nothing further was done with the eluates from these zones.

Placenta

The ether extract of the placenta weighed 33.3 mg and contained 381,350 dpm. It was chromatographed on a 40 g Celite column in system A and 10 ml fractions were collected. The gradient was applied after twelve hold-back volumes. Figure 16 shows a plot of cpm per fraction versus fraction number, and the radioactivity of various pooled fractions is shown in Table 23. At fraction 15, the automatic fraction collector stopped rotating unexpectedly and the eluate in the tube overflowed for some time. However, a sizable amount of radioactivity (139,700 dpm) was recovered from the floor of the turntable. This material was purified by Celite column chromatography using system A and a single peak of radioactivity was eluted in the fifth and sixth hold-back volumes. The fractions comprising this peak were pooled and yielded a thick oil which contained 59,450 dpm. The strippings from the column also consisted of a thick oil and had 73,400 dpm. Neither of these oily materials was processed further.

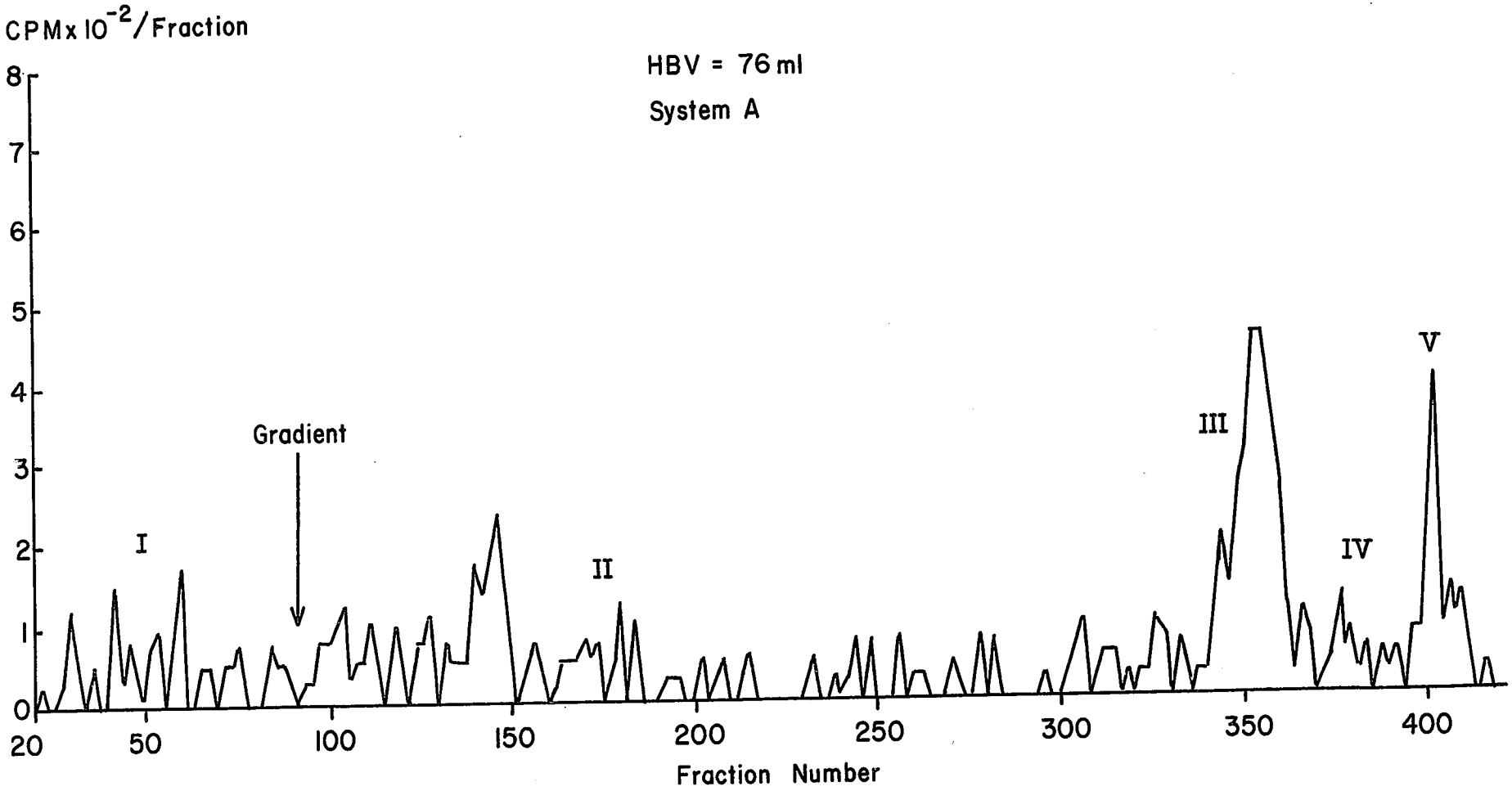


Fig. 16. Gradient Celite column chromatography of ether extract of placenta.

Table 23

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Ether Extract of the Placenta

Pool No.	Fractions Pooled	Weight (mg)	dpm
I	14-16	5.75	86,200
II	17-348	9.55	9,800
III	349-364	- *	8,600
IV	365-394	1.30	2,350
V	395-411	0.70	2,950
VI	412-553	3.15	600
Strippings		2.75	1,900

*Negligible

Pools IV, V and VI contained a small amount of radioactivity and were not processed further. The material from Pool I (86,200 dpm) was purified directly by paper chromatography in system J for 4 hours and then in system H for 8 hours. In both systems, a single symmetrical peak of radioactive material, which had the same mobility as pregnenolone run as a standard, was detected on scanning the chromatogram. The eluted material from the last chromatogram contained 61,000 dpm and an aliquot of this residue was mixed with 25.35 mg of carrier pregnenolone and the mixture was crystallized to constant specific activity as shown in Table 24.

The material from Pool II contained 9,800 dpm and was chromatographed on paper in system J for 3 1/2 hours. Two zones of radioactivity were detected on scanning the chromatogram. One zone was present at the origin and gave 5,600 dpm on elution, whereas the less polar area, which

migrated with the same mobility as authentic 3β-hydroxy-5α-pregnan-20-one, yielded 4,200 dpm. The latter material was combined with 10.30 mg of carrier 3β-hydroxy-5α-pregnan-20-one and the mixture was crystallized. After four crystallizations, the specific activities of the crystals and mother liquor were constant as shown in Table 25. The remainder of the crystals, which weighed 4.2 mg, were acetylated and the product was crystallized to constant specific activity as shown in Table 25. An infrared spectrum (KBr) of an aliquot of the last crystals was identical to that of 3β-acetoxy-5α-pregnan-20-one.

The material in Pool III (8,600 dpm) was purified by paper chromatography in system G for 4 hours. A scan of the chromatogram revealed a single peak of radioactive material which migrated with the same mobility as standard pregn-5-ene-3β,20β-diol. However, when the eluted radioactive material was chromatographed further in system J for 31 hours, the mobility of the unknown compound did not coincide with that of pregn-5-ene-3β,20β-diol, run as a standard. Nothing further was done with the eluted radioactive residue.

Table 24

Proof of Radiochemical Purity of Pregnenolone Isolated From the Ether Extract of the Placenta

	Specific Activity (dpm/mg)	
Crystallization	Crystals	Mother Liquor
1	1360	1370
2	1440	1410
3	1400	1410
Calculated	1440*	

*A total of 36,500 dpm was mixed with 25.35 mg of carrier pregnenolone prior to crystallization.

Table 25

Proof of Radiochemical Purity of 3β-Hydroxy-5α-pregnan-20-one Isolated From the Ether Extract of the Placenta

	Specific Activity (dpm/mg)			
	3β-Hydroxy-5α-pregnan-20-one		3β-Acetoxy-5α-pregnan-20-one	
Crystallization	Crystals	Mother Liquor	Crystals	Mother Liquor
1	93	2040	69	119
2	78	1250	72	70
3	73	200		
4	76	82		
Calculated	410*		76**	

*A total of 4,200 dpm was mixed with 10.30 mg of carrier 3β-hydroxy-5α-pregnan-20-one prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 3β-hydroxy-5α-pregnan-20-one and adjusting for the change in molecular weight.

Experiment 2 - Synthesis of 4-^{14}C-Labeled and Non-labeled 15α-Hydroxydehydroisoandrosterone

I. Synthesis of Non-labeled 15α-Hydroxydehydroisoandrosterone

A) Preparation of 3,15α-Diacetoxyandrost-3,5-dien-17-one

The synthesis of 15α-hydroxydehydroisoandrosterone was achieved starting with 15α-hydroxyandrostenedione. Only the first intermediate, 3,15α-diacetoxyandrost-3,5-dien-17-one, was purified and its structure established from its infrared and mass spectra. A solution of 103.9 mg of pure crystalline 15α-hydroxyandrostenedione in 10 ml of acetyl chloride and 5 ml of acetic anhydride was refluxed under nitrogen atmosphere for 90 minutes. The reagents were mainly removed in vacuo at 38°C, but the last traces of these solvents were removed by dissolving the crude enol acetate in approximately 20 ml of toluene and evaporating the solvent. This process was repreated until a crystalline residue was obtained. The crude product was then dissolved in 100 ml of ethyl acetate and this solution was washed three times with water; the organic phase was dried over Na_2SO_4, filtered and evaporated. The residue was crystallized from acetone-hexane mixtures and yielded 69.1 mg of crystals, mp 156-160°C. This material is stable only in the dry crystalline form and decomposes after a few days when kept in methanol, acetone or benzene. Infrared analysis (CS_2) of this compound gave a spectrum (Figure 17) which was compatible with the structure of 3,15α-diacetoxyandrost-3,5-dien-17-one. The spectrum showed the absence of a hydroxyl band, a strong band at 1740 cm^{-1} corresponding to both the 17-ketone and acetate groups, weak bands at 1670 cm^{-1} and 1640 cm^{-1} characterizing the $\Delta^{3,5}$-diene-3-acetate group, and a strong acetate band between 1250 and 1215 cm^{-1}. The mass

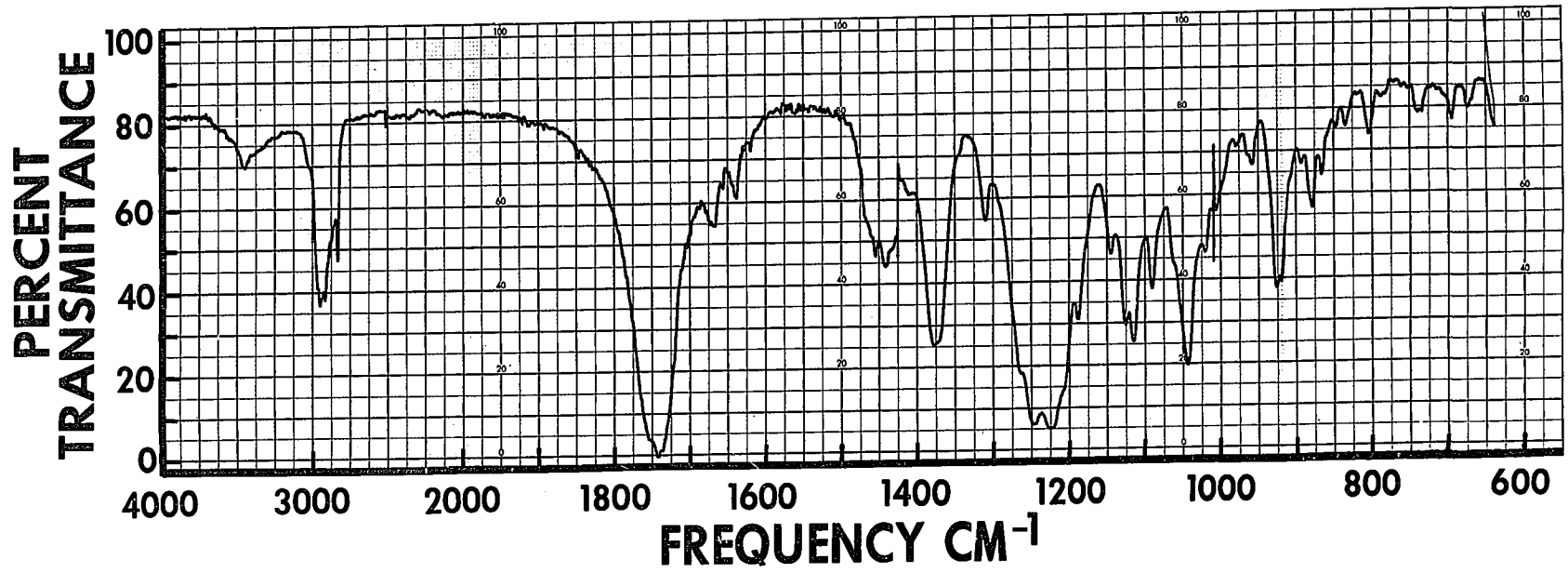


Fig. 17. Infrared Spectrum (CS_2) of 15α-Hydroxyandrostenedione enol acetate.

spectrum of the enol acetate indicated that it had a molecular weight of 386, which is consistent with the formula $C_{23}H_{30}O_5$. Significant heavy ions in the mass spectrum were observed at m/e 386 (M^+, 11%), 344 (M^+-42, 65%), 284 [M^+-(42+60), 26%] and 269 [M^+-(102+15), 8%]. These data indicated that the compound synthesized from 15α-hydroxyandrostenedione was 3,15α-diacetoxyandrost-3,5-dien-17-one.

B) Preparation of 15α-Hydroxydehydroisoandrosterone

In the actual synthesis of 15α-hydroxydehydroisoandrosterone, a total of 390 mg of 15α-hydroxyandrostenedione was converted to the enol acetate as described above. The crude enol acetate was dissolved in approximately 5 ml of benzene and to this solution were added 50 ml of ethylene glycol and 50 ml of p-toluenesulfonic acid. The mixture was stirred with a magnetic stirrer and the flask was connected to a high vacuum distillation unit. The temperature was raised to 38°C and the pressure was lowered to approximately 0.1 micron. The stirring and heating was continued for a period of 4 1/2 hours, and during this period a few drops of ethylene glycol distilled over. The reaction product (slurry) was dissolved in 500 ml of ethyl acetate and this solution was washed with water, dried over Na_2SO_4, filtered, and the ethyl acetate was evaporated.

The crude product from the last reaction was reduced with $NaBH_4$ and $LiAlH_4$. The ketal was first dissolved in 1 ml of benzene and 50 ml of 95% ethanol and chilled to 4-5°C, and to this solution was added dropwise a cold solution of 1 g of $NaBH_4$ in 50 ml of 95% ethanol. The reaction mixture was stirred for 15-17 hours at room temperature. Excess $NaBH_4$ was destroyed with acetic acid, and then the solvents were evaporated under vacuum. The residue was dissolved in 500 ml of ethyl acetate and it was

washed with water, dried over Na_2SO_4 and filtered. After evaporating the ethyl acetate, the reduced product was dissolved in 100 ml of dry ether and 300 mg of $LiAlH_4$ was added. The mixture was stirred for 1 hour at room temperature and then refluxed for 30 minutes. The excess reagent was destroyed by adding water and acetic acid, and the ether was evaporated. A neutral extract was prepared by extraction of the product with ethyl acetate as described above.

The product from the last reaction was hydrolyzed, resulting in the formation of 15α-hydroxydehydroisoandrosterone. The crude extract was dissolved in 300 ml of acetone and to this solution was added 500 mg of p-toluenesulfonic acid in 24 ml of water. The mixture was refluxed for 4 hours, the acetone was evaporated, the residual aqueous solution was neutralized with a saturated solution of sodium bicarbonate and the product was extracted with ethyl acetate. The organic phase was washed with water, dried over Na_2SO_4, filtered and the solvent was evaporated. The crude residue weighed 297.5 mg and was purified by thin-layer chromatography using system Z. The major Oertel positive zone (R_f 0.45) was eluted and the residue obtained was crystallized twice from acetone-hexane mixtures, yielding 25 mg of crystals, mp 223-224°C. The infrared spectrum (KBr) of this material (Figure 18) showed the presence of a hydroxyl band at 3300 cm^{-1} and a 17-ketone band at 1740 cm^{-1}. The mass spectrum indicated that the molecular weight of this compound was 304, which agrees with the formula $C_{19}H_{28}O_3$. Significant heavy ions in this spectrum were observed at m/e 304 (M^+, 57%), 289 (M^+-15, 6%), 286 (M^+-18, 54%), 271 [M^+-(15+18), 33%], 253 [M^+-(36+15), 58%] and 227 [M^+-(51+26), 40%]. Anal. Calcd for $C_{19}H_{28}O_3$: C, 74.96; H, 9.28. Found: C, 74.86; H, 9.28. These data were

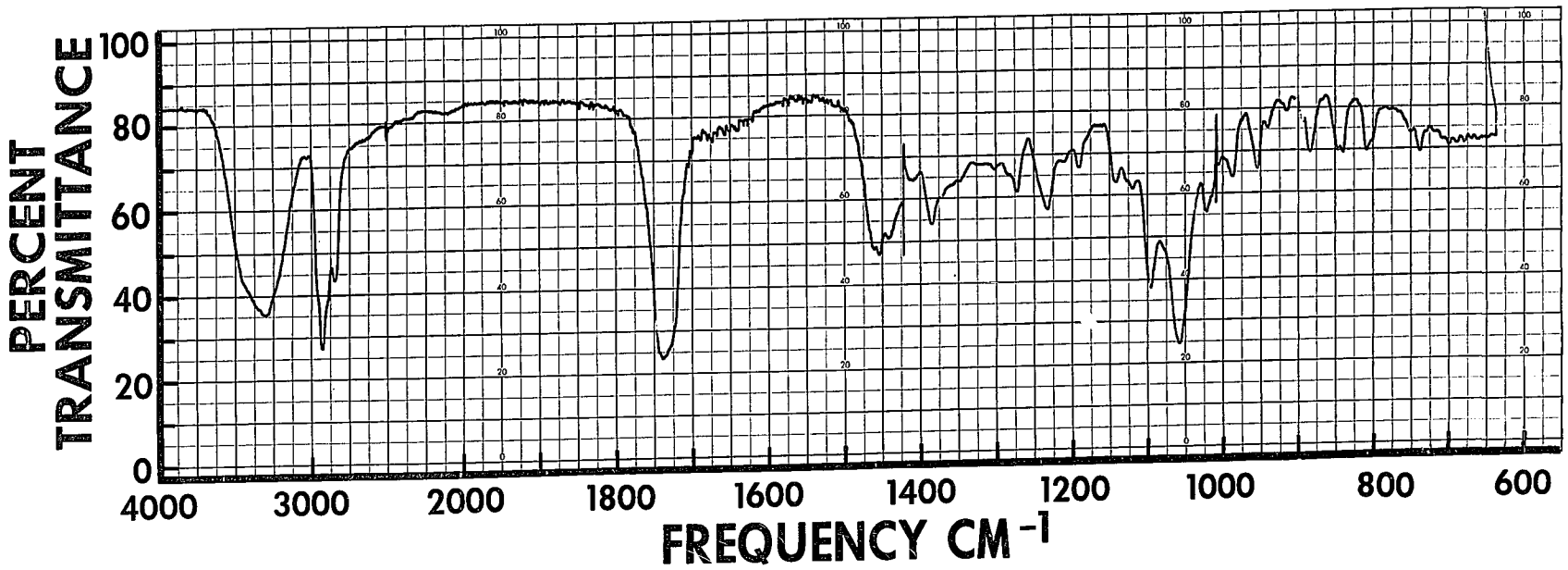


Fig. 18. Infrared Spectrum (KBr) of 15α-Hydroxydehydroisoandrosterone.

compatible with the structure assigned to the final product of the sequence of reactions just described.

C) Enzymatic Conversion of 15α-Hydroxydehydroisoandrosterone to 15α-Hydroxyandrostenedione

Further confirmation of the structure of 15α-hydroxydehydroisoandrosterone was obtained by converting it enzymatically to 15α-hydroxyandrostenedione. A total of 500 μg of 15α-hydroxydehydroisoandrosterone was dissolved in 0.2 ml of propylene glycol and to this solution were added 5 ml of 0.067 M phosphate buffer (pH 8.9), 10 mg of βNAD and 10 mg of 3β-hydroxysteroid dehydrogenase-Δ^5-isomerase. The mixture was incubated for 2 hours at 25°C in air. The incubation product was extracted twice with 25 ml of ethyl acetate and the pooled ethyl acetate was washed with water, dried over Na_2SO_4, and the organic solvent was evaporated. The residue obtained weighed 1.30 mg and was purified by chromatography on paper using system M for 4 hours. A major ultraviolet absorbing material having the mobility of 15α-hydroxyandrostenedione was eluted and weighed 0.45 mg. The infrared spectrum (KBr) of this material was identical with that of 15α-hydroxyandrostenedione.

II. Synthesis of Derivatives of 15α-Hydroxydehydroisoandrosterone

A) Preparation of 15α-Hydroxydehydroisoandrosterone diacetate

A total of 34 mg of 15α-hydroxydehydroisoandrosterone was acetylated as described earlier and the reaction product was purified by chromatography on a 5 g alumina column. Elution with 60% benzene in ligroin B gave a residue which after two crystallizations from ethanol gave 24 mg of crystals, mp 186-188°C. The infrared spectrum (KBr) of

this material (Figure 19) showed the absence of a hydroxyl band, the presence of a band at 1740 cm^{-1}, representing both the 17-ketone and acetate groups, and an acetate band at 1244 cm^{-1}. No parent ion peak was observed in the mass spectrum of this compound, however, the spectrum had a peak at m/e 328, which resulted from loss of acetic acid (M^+-60). Significant heavy ions were observed at m/e 328 (M^+-60, 5.4%), 268 (M^+-120, 100%) and 253 [M^+-(120+15), 29%]. Anal. Calcd for $C_{23}H_{32}O_5$: C, 71.10; H, 8.33. Found: C, 70.98; H, 8.33. The NMR spectrum of this compound (Figure 20) indicated that the chemical shifts for the two methyl groups at C_{18} and C_{19} occurred at 0.96 and 1.05 ppm, respectively. These values are in agreement with the calculated values of 0.97 and 1.06 ppm from Zürcher's Tables (66). The signals for the two acetate methyls were present at 2.03 and 2.05 ppm and the signals for the C-6 vinyl proton, the C-15 proton and the C-3 proton occurred at 5.36, 5.18 and 4.58, respectively; NMR δ 0.96 (singlet, 3H, 18-CH_3), 1.05 (singlet, 3H, 19-CH_3), 2.02, 2.05 (singlets, 3H, acetate methyls), 4.58 (multiplet, 1H, 3α-H), 5.18 (multiplet, 1H, 15βH) and 5.36 (multiplet, 1H, C-6 olefinic H). The NMR data therefore indicated that the acetylated product was 15α-hydroxydehydroisoandrosterone diacetate. This conclusion was reinforced by the data from the infrared, mass and elemental analyses.

B) Preparation of Androst-5-ene-3β,15α,17β-triol

A total of 28.4 mg of 15α-hydroxydehydroisoandrosterone was reduced with $NaBH_4$ as described previously. The reaction product was purified by thin-layer chromatography in system A1. The major Oertel positive zone (R_f 0.25) was eluted and the residue obtained was crystallized twice from methanol-hexane mixtures, yielding 14.9 mg of crystals,

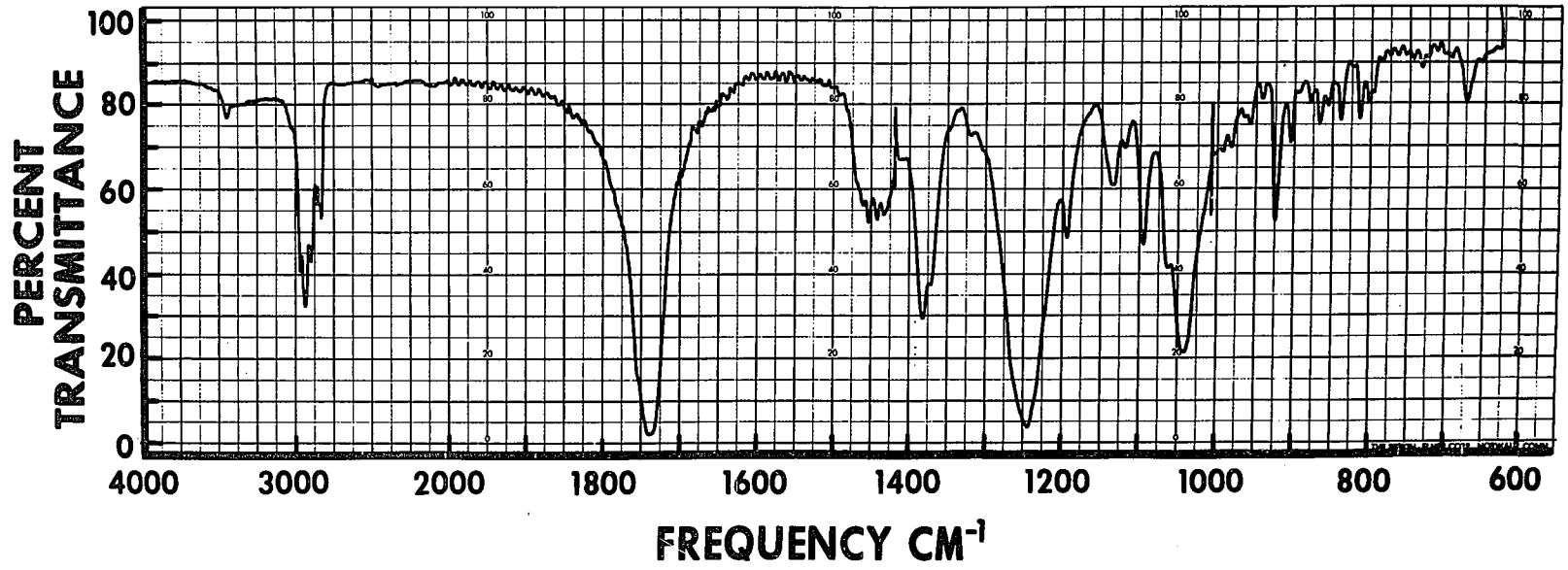


Fig. 19. Infrared Spectrum (KBr) of 15α-Hydroxydehydroisoandrosterone diacetate.

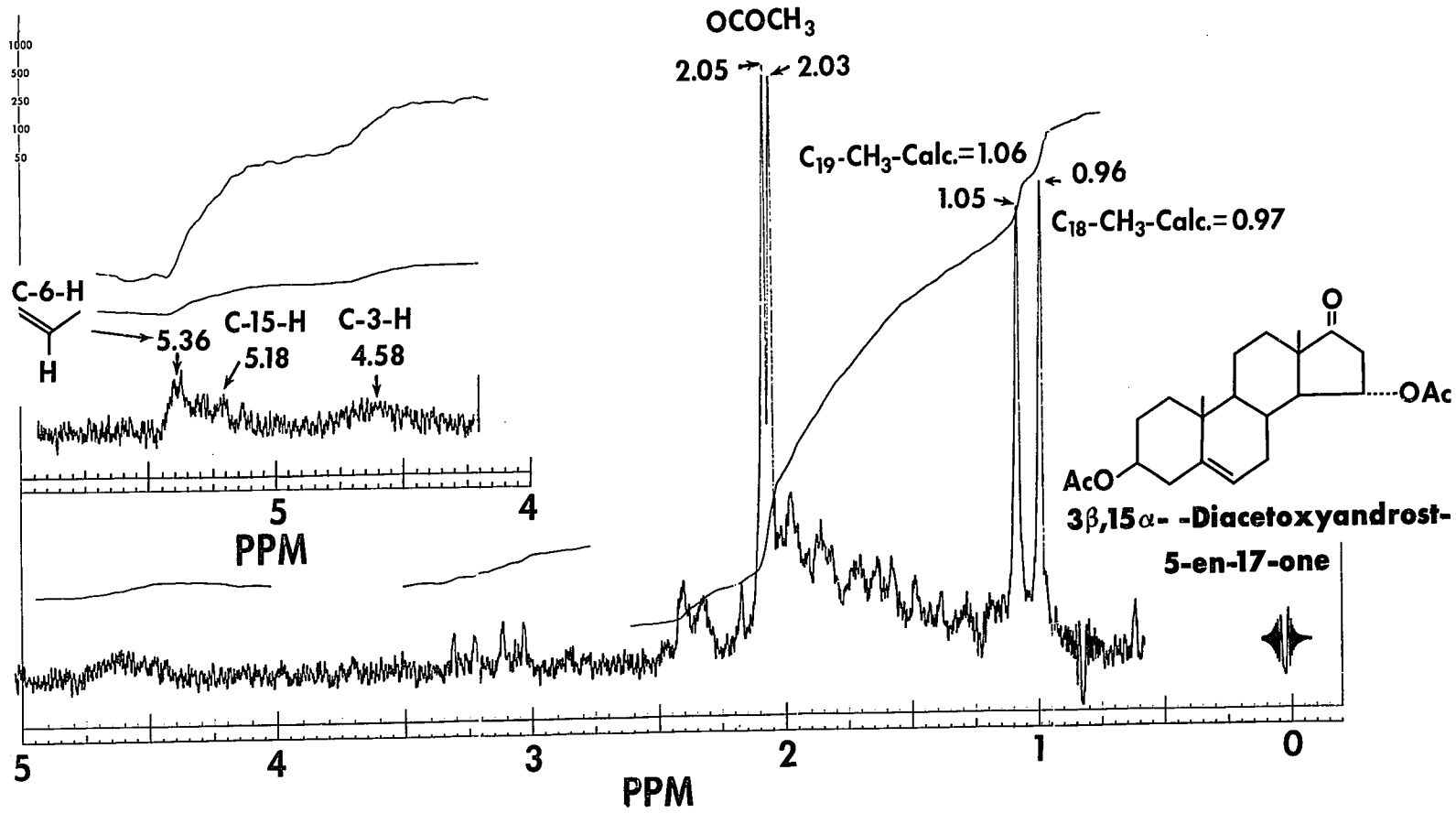


Fig. 20. NMR Spectrum of 15α-Hydroxydehydroisoandrosterone diacetate.

mp 271-274°C. Infrared analysis (KBr) of an aliquot of these crystals gave a spectrum (Figure 21) which was characterized by the absence of a 17-ketone band at 1740 cm^{-1}, the presence of a strong hydroxyl absorption at 3300 cm^{-1} and a band at 1668 cm^{-1} representing the C-5 double bond. Mass spectral analysis indicated that the compound had a molecular weight of 306, which is compatible with the structural formula $C_{19}H_{30}O_3$. Significant heavy ions were observed at m/e 306 (M^+, 25.7%), 288 (M^+-18, 100%), 270 (M^+-36, 23.0%), 255 [M^+-(36+15), 90.5%] and 229 [M^+-(51+26), 51.3%]. These data indicated that the reduced compound could therefore be assigned the structure of androst-5-ene-3β,15α,17β-triol.

C) Preparation of Androst-5-ene-3β,15α,17β-triol-triacetate

A total of 23.6 mg of androst-5-ene-3β,15α,17β-triol was acetylated as described earlier and the product was purified on a 5 g alumina column. Elution with 40% benzene in hexane gave an oily residue which on crystallization from ethanol yielded 20.9 mg of crystals, mp 121-123°C. The infrared spectrum (KBr) of this material (Figure 22) was characterized by the absence of a hydroxyl band and the presence of bands at 1735 and 1238 cm^{-1} representing the acetate groups. Mass spectroscopy gave a spectrum which had no parent ion peak at m/e 432, however, a peak was present at m/e 372 which resulted from loss of acetic acid (M^+-60). Significant heavy ions were observed at m/e 372 (M^+-60, 14.1%), 312 (M^+-120, 100%), 252 (M^+-180, 23.1%) and 237 [M^+-(180+15), 25.6%]. These results are consistent with the structure of androst-5-ene-3β,15α,17β-triol triacetate assigned to the acetylated product.

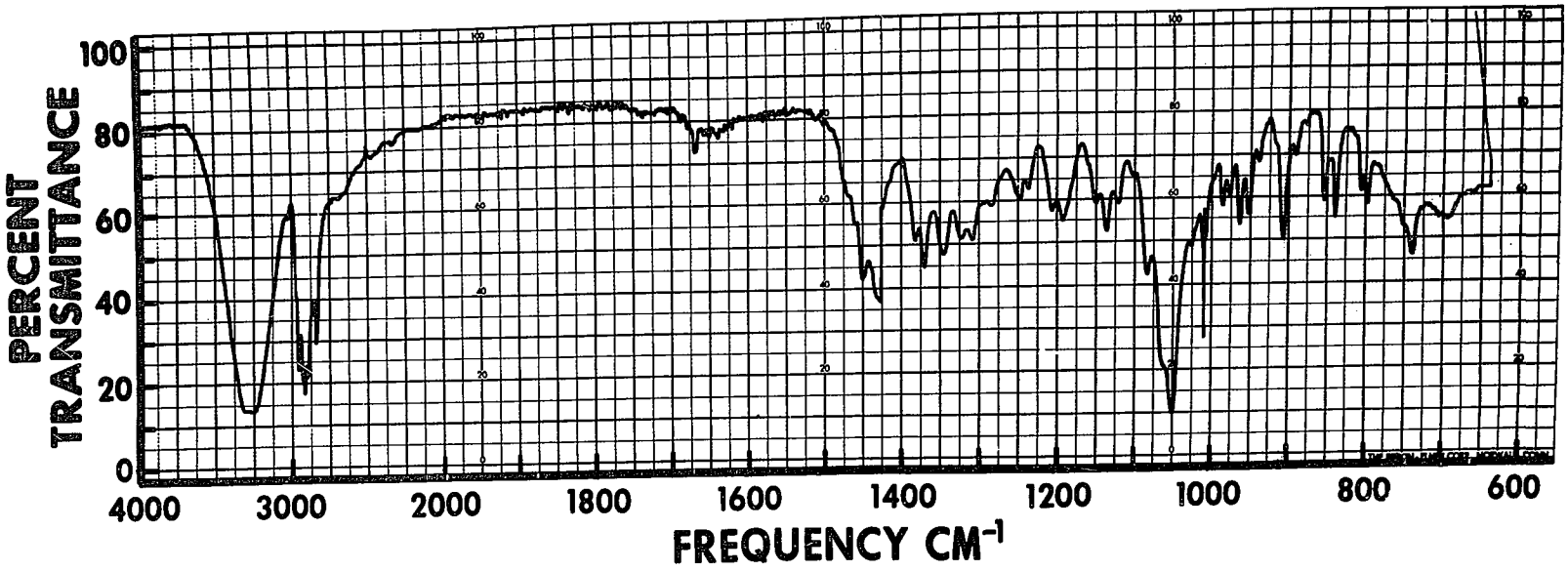


Fig. 21. Infrared Spectrum (KBr) of Androst-5-ene-3β,15α,17β-triol.

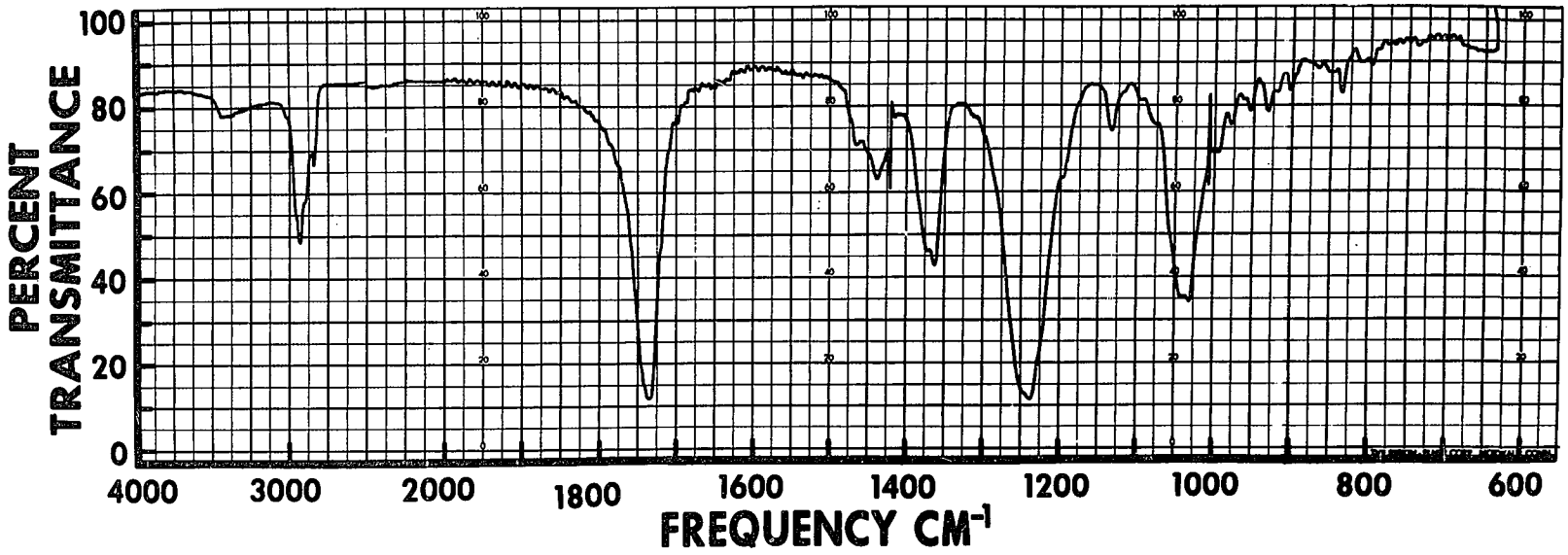


Fig. 22. Infrared Spectrum (KBr) of Androst-5-ene-3β,15α,17β-triol triacetate.

III. Synthesis of 4-^{14}C-15α-Hydroxydehydroisoandrosterone

The procedure described above for the synthesis of non-labeled 15α-hydroxydehydroisoandrosterone was also used for the preparation of the labeled steroid. A total of 1.6 x 10^8 dpm of ^{14}C-15α-hydroxyandrostenedione (specific activity 5.0 x 10^4 dpm/μg) was refluxed with 2 ml of acetic anhydride and 4 ml of acetyl chloride under nitrogen atmosphere for 45 minutes. The reagents were removed mainly in vacuo at 38°C, but the last traces were removed by dissolving the crude enol acetate in a small volume of benzene and evaporating the solvent. This process was repeated until the odor of the reagents could not be detected. The crude enol acetate obtained was dissolved in 25 ml of ethyl acetate and this solution was washed with water, dried over Na_2SO_4, filtered and evaporated under vacuum. The residue was dissolved in a few drops of benzene and to this solution were added 1 ml of ethylene glycol and 1 mg of p-toluenesulfonic acid. The mixture was stirred with a magnetic stirrer and the flask was connected to a high vacuum distillation unit as described previously; the stirring and heating (38°C) was continued for only 2 hours. The reaction product was dissolved in 50 ml of ethyl acetate and a neutral extract was prepared as described above. The extract was dissolved in 1 ml of 95% ethanol and chilled to 4-5°C. To this solution was added 10 mg of $NaBH_4$ and the reaction mixture was stirred for 20 hours at room temperature. Excess reagent was destroyed by the addition of acetone and a few drops of acetic acid, and the solvents were evaporated under vacuum. The product obtained was extracted twice with 30 ml of ethyl acetate and a neutral extract was prepared in the manner set forth above. The residue (1.2 x 10^8 dpm) was dissolved in 5 ml of ether, and 5 mg of $LiAlH_4$ was added to the solution.

The mixture was stirred at room temperature for 30 minutes and then refluxed for 30 minutes. Excess reagent was destroyed by adding three drops of acetic acid and 20 ml of water, and the ether was evaporated. The product was extracted twice from the aqueous phase remaining, using 60 ml of ethyl acetate, and a neutral extract was prepared as described previously. The residue (1.0×10^8 dpm) was dissolved in 2 ml of acetone and to this solution was added 1 mg of p-toluenesulfonic acid in 0.2 ml of water. The mixture was refluxed for 2 hours and then the acetone was evaporated and a neutral extract was prepared as described above.

The residue obtained from the last reaction weighed 7.0 mg and contained 9.1×10^7 dpm, and it was purified by chromatography on paper sequentially in systems P (7 1/2 hours), O (50 hours), M (4 hours), G (70 hours) and Q (96 hours). In each of these systems the radioactive material corresponding in mobility to 15α-hydroxydehydroisoandrosterone, run as a standard, was eluted, and a total of 1.3×10^7 dpm was recovered from the last chromatogram. Radiochemical purity at this stage was determined by isotope dilution as shown in Table 26. The final crystals and mother liquor were combined and had a weight of 8.0 mg. They were acetylated and the product was chromatographed on a 3 g alumina column. Elution with 60% benzene in ligroin B gave a residue weighing 10.5 mg and containing 32,250 dpm; this material was crystallized to constant specific activity as shown in Table 26. The infrared spectrum (KBr) of this compound was identical to that of standard 15α-hydroxydehydroisoandrosterone diacetate. The overall yield of ^{14}C-15α-hydroxydehydroisoandrosterone, starting with ^{14}C-15α-hydroxyandrostenedione, was 8%, and the purity of the former compound was found to be over 97%.

Table 26

Proof of Radiochemical Purity of ^{14}C-15α-Hydroxydehydroisoandrosterone Synthesized Chemically From ^{14}C-15α-Hydroxyandrostenedione

	Specific Activity (dpm/mg)			
	4-^{14}C-15α-Hydroxydehydroisoandrosterone		4-^{14}C-15α-Hydroxydehydroisoandrosterone diacetate	
Crystallization	Crystals	Mother Liquor	Crystals	Mother Liquor
1	3870	3970	3040	3030
2	3870	3830	3100	3050
3	3810	3800		
Calculated	3940*		2980**	

*A total of 66,350 dpm was mixed with 16.85 mg of carrier 15α-hydroxydehydroisoandrosterone prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 15α-hydroxydehydroisoandrosterone and adjusting for the change in molecular weight.

Experiment 3 - Isolation of 15α-Hydroxydehydroisoandrosterone From Human Late Pregnancy Urine

I. Isolation of 15α-Hydroxydehydroisoandrosterone From a 15-Day Pool of Pregnancy Urine

Fifteen 24-hour urine collections were obtained from normal subjects in the third trimester of pregnancy, and the pooled urine had a volume of 18.7 liters. To this urine pool was added a total of 5.65×10^5 dpm of ^{14}C-15α-hydroxydehydroisoandrosterone (specific activity 4.55×10^4 dpm/μg) as a recovery marker and the urinary conjugates were hydrolyzed using Glusulase as described earlier. The neutral extract obtained had a weight of 3.87 g and contained 5.37×10^5 dpm. It was chromatographed on a 400 g silica gel column which was developed with increasing concentrations of ethanol in methylene dichloride. The effluent from the column was collected in 20 ml fractions at the rate of 80-90 ml/hour. A single peak of radioactivity was eluted with 4% ethanol in methylene dichloride, yielding a residue which weighed 226 mg and contained 5.10×10^5 dpm. It was further purified on a 120 g Celite column (HBV = 220 ml) using system D. Ten ml fractions were collected and a single peak of radioactive material was eluted in the sixth to eighth hold-back volumes. The residue obtained from this peak weighed 41 mg and contained 4.62×10^5 dpm and was further purified by paper chromatography sequentially in systems M (4 hours), O (60 hours), P (6 1/2 hours) and N (4 days). In each system, a single peak of radioactivity, which had the same mobility as 15α-hydroxydehydroisoandrosterone, run as a standard, was detected on scanning the chromatogram. Elution of the material from the radioactive zone of the last chromatogram yielded a residue which weighed 0.70 mg and contained 2.94×10^5 dpm. It was then purified by thin-layer chromatography in

system A2 using two developments, and the single radioactive zone detected, migrated with the same mobility as 15α-hydroxydehydroisoandrosterone and, on elution, gave a residue weighing 0.50 mg and containing 2.73×10^5 dpm.

A total of 4.35×10^4 dpm of the eluted material was acetylated with ^3H-acetic anhydride (Batch No. 1) in the manner described earlier. After extraction, the acetylated product contained 3.46×10^6 dpm of ^3H and 4.09×10^4 dpm of ^{14}C ($^3H/^{14}C = 84.7$). The crude product was purified by paper chromatography sequentially in systems J (3 1/2 hours), I (4 hours) and V (4 hours). In each system, the major peak of radioactivity migrated with the same mobility as authentic 15α-hydroxydehydroisoandrosterone diacetate, and elution of this radioactive material from the last chromatogram gave 8.54×10^5 dpm of ^3H and 2.23×10^4 dpm of ^{14}C ($^3H/^{14}C = 38.3$). The eluted material was combined with 25.15 mg of carrier 15α-hydroxydehydroisoandrosterone diacetate and the mixture was percolated through a 3 g alumina column. Elution with 60% benzene in ligroin B gave an oily residue which weighed 24.35 mg and contained 6.99×10^5 dpm of ^3H and 1.72×10^4 dpm of ^{14}C ($H^3/C^{14} = 40.5$). This fraction was then crystallized to a constant $^3H/^{14}C$ ratio as shown in Table 27. The final crystals, which weighed 13.0 mg were reduced with $NaBH_4$ and the crude product was applied on a 2 g alumina column. Elution with 0.5% ethanol in benzene gave a residue which contained 3.13×10^4 dpm of ^3H and 6.65×10^3 dpm of ^{14}C ($^3H/^{14}C = 4.71$). This material was crystallized from acetone-ligroin B mixtures and it was established that the compound was radiochemically pure as shown in Table 27. Infrared analysis (KBr) of an aliquot of the final crystals gave a spectrum which was compatible with the structure of 3β,15α-diacetoxyandrost-5-en-17β-ol. The spectrum showed the presence of a

Table 27

Proof of Radiochemical Purity of 15α-Hydroxydehydroisoandrosterone Isolated From a 15-Day Pool of Pregnancy Urine

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxydehydroisoandrosterone diacetate						
1	4,080	590	6.90	218,260	1,360	159.0
2	2,940	570	5.15	38,080	810	47.0
3	2,810	560	4.96	4,230	580	7.28
4	2,830	570	4.93	3,430	570	6.00
5	2,780	570	4.88	2,860	560	5.11
Calculated		890*				
3β,15α-Diacetoxyandrost-5-en-17β-ol						
1	2,740	570	4.82	2,220	430	5.11
2	2,730	570	4.78	2,690	570	4.73
Calculated	2,780**	570**				

*A total of 22,300 dpm of ^{14}C was mixed with 25.15 mg of carrier 15α-hydroxydehydroisoandrosterone diacetate.

**This value was calculated by using the specific activity of the final crystals of 15α-hydroxydehydroisoandrosterone diacetate and adjusting for the change in molecular weight.

hydroxyl band at 3450 cm^{-1}, acetate bands at 1735 and 1240 cm^{-1} and a weak band at 1668 cm^{-1} representing the C-5 double bond. Using the final $^{3}H/^{14}C$ ratio of the last crystals of the derivative ($^{3}H/^{14}C$ = 4.78) and the specific activity of the ^{3}H-acetic anhydride (Batch No. 1), the specific activity of the urinary 15α-hydroxydehydroisoandrosterone was calculated to be 9.34 x 10^{3} dpm/μg.

An aliquot from the stock solution of the ^{14}C-15α-hydroxydehydroisoandrosterone which was added to the urine pool was acetylated with ^{3}H-acetic anhydride (Batch No. 1) as described earlier and the acetylated product was purified by paper chromatography sequentially in systems J (3 1/2 hours), I (4 hours) and V (4 hours). In each system, the major peak of radioactivity had the same mobility as 15α-hydroxydehydroisoandrosterone diacetate, run as a standard. Elution of this radioactive material from the last chromatogram gave 1.30 x 10^{5} dpm of ^{3}H and 1.02 x 10^{5} dpm of ^{14}C ($^{3}H/^{14}C$ = 1.27). It was then mixed with 18.90 mg of carrier 15α-hydroxydehydroisoandrosterone diacetate and the mixture was percolated through a 2 g alumina column. Elution with 60% benzene in ligroin B yielded a residue which weighed 18.9 mg and contained 1.03 x 10^{5} dpm of ^{3}H and 8.92 x 10^{4} dpm of ^{14}C ($^{3}H/^{14}C$ = 1.15). This material was crystallized to constant specific activity as shown in Table 28. Using the $^{3}H/^{14}C$ ratio of the final crystals ($^{3}H/^{14}C$ = 0.98) and the specific activity of the ^{3}H-acetic anhydride (Batch No. 1), the specific activity of the ^{14}C-15α-hydroxydehydroisoandrosterone added to the urine was calculated to be 4.55 x 10^{4} dpm/μg. Therefore, the total of 5.65 x 10^{5} dpm of ^{14}C-15α-hydroxydehydroisoandrosterone added to the urine represented 12.40μg. From this value and the final specific activities of the

^{14}C-15α-hydroxydehydroisoandrosterone added to the urine and the isolated 15α-hydroxydehydroisoandrosterone, it was calculated that the amount of the latter compound excreted in the urine was 3.20 μg/day.

An attempt to obtain an infrared spectrum of the isolated urinary 15α-hydroxydehydroisoandrosterone was unsuccessful. The remaining portion of the eluate obtained from the last chromatogram before acetylation of the isolated 15α-hydroxydehydroisoandrosterone with ^{3}H-acetic anhydride was purified further by paper chromatography in systems G (96 hours) and Q (103 hours) and by thin-layer chromatography in system A3. A single zone of radioactivity, which migrated with the same mobility as authentic 15α-hydroxydehydroisoandrosterone, was detected in each system, and the eluted material from the thin-layer plate weighed 30 μg (on a microbalance) and contained 9.66×10^{4} dpm. Infrared analysis of this material (micro KBr) gave a poorly defined spectrum which showed the presence of a hydroxyl band at 3300 cm^{-1} and a ketone band at 1740 cm^{-1}.

Table 28

Determination of the Specific Activity of ^{14}C-15α-Hydroxydehydroisoandrosterone Added to the 15-Day Pool of Pregnancy Urine

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	^{3}H/^{14}C	^{3}H	^{14}C	^{3}H/^{14}C
1	4,460	4,510	0.99	20,070	8,130	2.47
2	4,350	4,440	0.98	5,730	5,580	1.03
3	4,270	4,340	0.98	4,110	4,360	0.94
Calculated		5,400*				

*A total of 101,900 dpm of ^{14}C was mixed with 18.90 mg of carrier 15α-hydroxydehydroisoandrosterone diacetate prior to crystallization.

II. Isolation of 15α-Hydroxydehydroisoandrosterone From a 5-Day Pool of Pregnancy Urine

In order to confirm the isolation of 15α-hydroxydehydroisoandrosterone, a second study was performed using a 5-day urine collection obtained from normal subjects in the third trimester of pregnancy. The pooled urine had a volume of 7.6 liters and to it was added a total of 1.51×10^5 dpm of ^{14}C-15α-hydroxydehydroisoandrosterone (specific activity 4.93×10^4 dpm/μg). After hydrolysis of the urinary conjugates with Glusulase, the neutral extract obtained weighed 1.30 g and contained 1.30×10^5 dpm. It was first purified by chromatography on a 140 g silica gel column using increasing concentrations of ethanol in methylene dichloride and 10 ml fractions were collected. A single peak of radioactivity was eluted with 4% ethanol in methylene dichloride, giving a residue with a weight of 76.6 mg and 1.24×10^5 dpm. This material was then chromatographed on a 75 g Celite column (HBV = 150 ml) in system D. Ten ml fractions were collected and a radioactive peak was obtained in the eight and ninth hold-back volumes, yielding a residue which weighed 3.70 mg and contained 1.18×10^5 dpm. This material was purified by paper chromatography in system M for 5 hours and system O for 68 1/2 hours. A single zone of radioactivity, which migrated with the same mobility as standard 15α-hydroxydehydroisoandrosterone was detected in both systems.

At this stage, a different approach was used to determine the urinary excretion rate of 15α-hydroxydehydroisoandrosterone for reasons which will be discussed later. The eluted radioactive material from the last chromatogram had a weight of 1.10 mg and contained 1.06×10^5 dpm. Half of this residue was reduced with $NaBH_4$ and the product was chromatographed on paper in system M for 41 hours. A peak of radioactivity, which

had the same mobility as androst-5-ene-3β,15α,17β-triol, run as a standard, was detected on scanning the chromatogram and elution of the radioactive material yielded 3.67 x 10^5 dpm. This material was acetylated with ^{3}H-acetic anhydride (Batch No. 2) and the product was mixed with 21.80 mg of carrier androst-5-ene-3β,15α,17β-triol triacetate. The mixture was chromatographed on a 5 g alumina column and elution with 40% benzene in hexane gave a residue which weighed 23.05 mg and contained 8.32 x 10^5 dpm of ^{3}H and 2.95 x 10^4 dpm of ^{14}C (^{3}H/^{14}C = 28.1). It was crystallized to constant ^{3}H/^{14}C ratio as shown in Table 29. The specific activity of the urinary 15α-hydroxydehydroisoandrosterone was calculated to be 1.27 x 10^4 dpm/μg.

An aliquot of the stock solution of ^{14}C-15α-hydroxydehydroisoandrosterone which was added to the urine pool was reduced with $NaBH_4$ and the product was purified by paper chromatography in system M for 40 hours. The peak of radioactivity corresponding in mobility to androst-5-ene-3β,15α,17β-triol, run as a standard, was eluted and the eluted radioactive material contained 4.18 x 10^5 dpm. It was acetylated with ^{3}H-acetic anhydride (Batch No. 2), the product was mixed with 13.45 mg of carrier androst-5-ene-3β,15α,17β-triol triacetate and the mixture was purified by chromatography on a 3 g alumina column. The residue eluted with 40% benzene in hexane weighed 13.30 mg and contained 3.42 x 10^5 dpm of ^{3}H and 3.18 x 10^4 dpm of ^{14}C (^{3}H/^{14}C = 10.7). This material was crystallized to constant specific activity as shown in Table 30 and the specific activity of the ^{14}C-15α-hydroxydehydroisoandrosterone added to the urine was calculated to be 4.93 x 10^4 dpm/μg. Thus, the amount of 15α-hydroxydehydroisoandrosterone added to the urine was 3.06 μg and the urinary excretion rate of this compound was computed to be 1.76 μg/day.

Table 29

Proof of Radiochemical Purity of 15α-Hydroxydehydroisoandrosterone Isolated From a 5-Day Pool of Pregnancy Urine

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
1	18,230	1,300	14.0	129,200	1,360	94.7
2	12,470	1,330	9.4	43,530	1,360	32.1
3	11,630	1,300	9.0	19,040	1,350	14.1
4	11,940	1,330	9.0	14,840	1,290	11.5
5	11,410	1,300	8.8	12,830	1,280	10.0
6	11,420	1,290	8.9	11,260	1,260	8.9
Calculated		1,280*				

*This value is based on the ^{14}C dpm and weight of the fraction eluted from the alumina column prior to crystallization.

Table 30

Determination of the Specific Activity of 4-^{14}C-15α-Hydroxydehydroisoandrosterone Added to the 5-Day Pool of Pregnancy Urine

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
1	7,990	2,360	3.4	55,630	2,600	21.4
2	5,480	2,380	2.3	13,240	2,370	5.6
3	5,240	2,380	2.2	6,030	2,340	2.6
4	5,280	2,330	2.3	5,430	2,280	2.4
Calculated		2,390*				

*This value is based on the ^{14}C dpm and weight of the fraction eluted from the alumina column prior to crystallization.

Experiment 4 - Metabolism of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone in Pregnant Subjects

I. Subject TL

A normal 22-year old subject (TL) in the 33rd week of pregnancy was injected intravenously with a mixture of 1.62×10^{7} dpm of ^{3}H-15α-hydroxyandrostenedione (specific activity 1.66×10^{6} dpm/μg) and 3.10×10^{6} dpm of ^{14}C-15α-hydroxydehydroisoandrosterone (specific activity 4.93×10^{4} dpm/μg), $^{3}H/^{14}C = 5.2$. Urine was collected for four days and the amount of radioactivity measured in each day's urine is shown in Table 31. After pooling the urine, the unconjugated steroids were extracted and were found to constitute less than 1% of the injected radioactivity as shown in Table 32. The conjugates in the urine were first solvolyzed and then hydrolyzed with β-glucuronidase, and the weight and radioactivity of the hydrolysates are shown in Table 32.

Table 31

Radioactivity Excreted in the Urine Following the Intravenous Injection of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone in Subject TL

Day	Volume (ml)	^{3}H (dpm)	% of I.D.*	^{14}C (dpm)	% of I.D.	$^{3}H/^{14}C$
1	900	1.02×10^{7}	62.9	1.60×10^{6}	51.6	6.4
2	550	5.92×10^{5}	3.6	6.68×10^{4}	2.1	8.9
3	760	2.54×10^{5}	1.5	2.28×10^{4}	0.7	11.1
4	1160	1.78×10^{5}	1.1	1.33×10^{4}	0.4	13.4
TOTAL	3370	1.12×10^{7}	69.1	1.70×10^{6}	54.8	6.6

*I.D. = Injected Dose

Table 32

Weight and Radioactivity of the Unconjugated and Conjugated Fractions From the Pregnancy Urine of Subject TL

Fraction	Weight (g)	^{3}H (dpm)	% of Radioactivity in Urine	^{14}C (dpm)	% of Radioactivity in Urine	$^{3}H/^{14}C$
Unconjugated	0.10	4.32×10^{4}	0.4	7.60×10^{3}	0.4	5.7
Sulfate	0.85	1.85×10^{6}	16.5	2.71×10^{5}	15.9	6.8
Glucosiduronate	1.10	7.09×10^{6}	63.3	1.37×10^{6}	80.6	5.2
TOTAL		8.98×10^{6}	80.2	1.65×10^{6}	97.0	5.4

The sulfate fraction (Table 32) was chromatographed on a 150 g silica gel column using increasing concentrations of ethanol in methylene dichloride, and the graph representing the radioactivity eluted versus fraction number is shown in Figure 23. The weight and radioactivity of the pooled fractions is shown in Table 33. The material in Pools VI, VII, VIII, XI, XII and XIII was further purified by paper chromatography, but the metabolites present in these pools could not be identified.

The fraction containing steroids excreted as glucosiduronates (Table 32) was chromatographed on a 250 g silica gel column using increasing concentrations of ethanol in methylene dichloride. The dpm per fraction plotted against fraction number is shown in Figure 24, and the weight and radioactivity of the pooled fractions are given in Table 34. The material in Pools II, VI and VIII was not processed further since it did not represent a peak of radioactivity, whereas the residues from Pools I and X are now being purified.

The residue of Pool III (Figure 24, Table 34) was further chromatographed on a 52 g Celite column (HBV = 104 ml) using system D. Five ml fractions were collected and a single radioactive peak was eluted in the third to fifth hold-back volumes. The material comprising this peak weighed 8.9 mg and contained 3.35×10^6 dpm of ^3H and 2.88×10^5 dpm of ^{14}C ($^3H/^{14}C = 11.7$). It was purified by paper chromatography in system O for 24 hours and in system M for 4 hours. In both systems, a single radioactive zone, which migrated with the same mobility as authentic 15α-hydroxyandrostenedione, was detected on scanning the chromatogram. The eluted material from the last chromatogram weighed 1.5 mg and contained

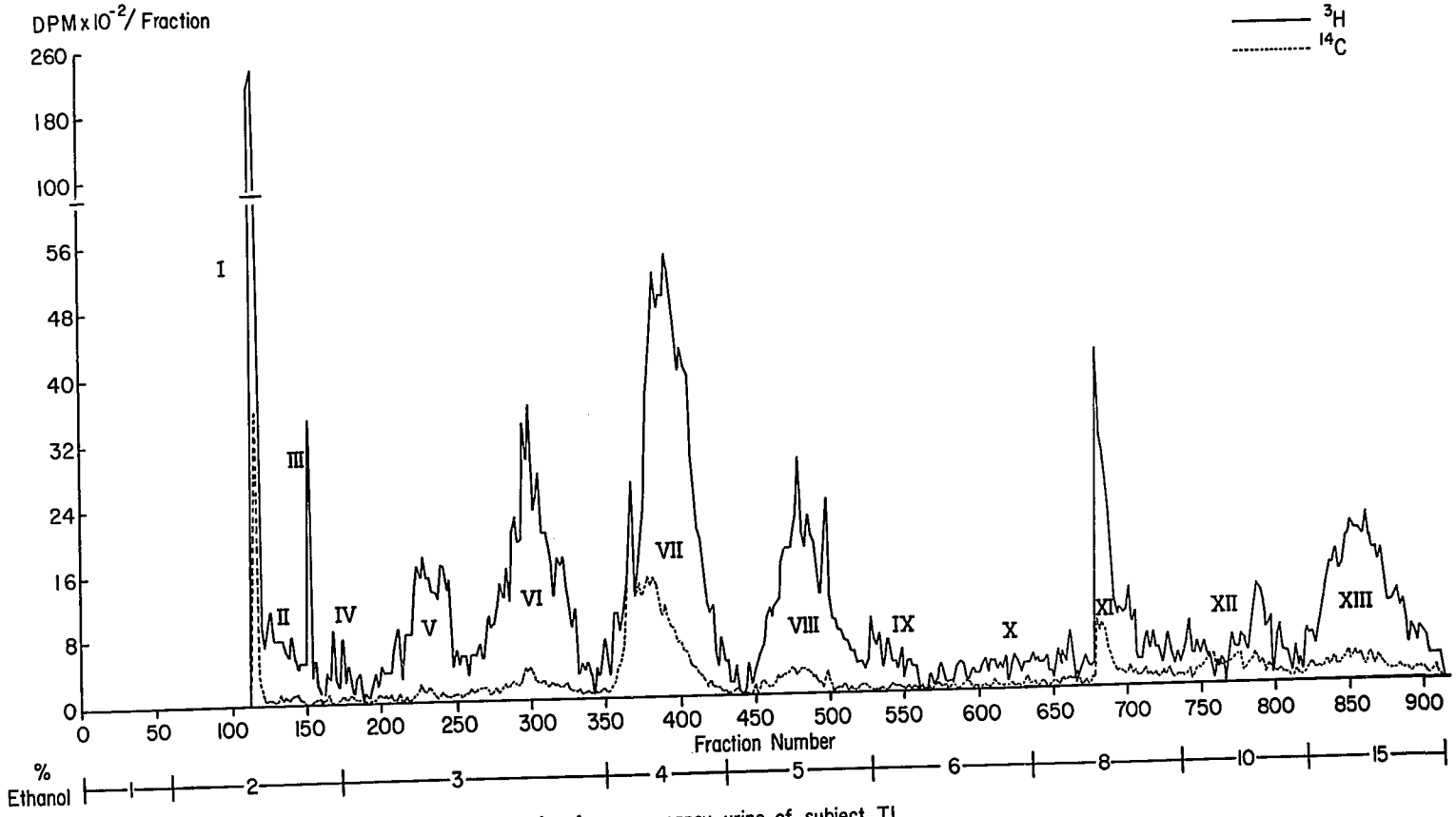


Fig. 23. Silica gel column chromatography of sulfate fraction from pregnancy urine of subject TL.

Table 33

Weight and Radioactivity of Pooled Fractions Obtained After Silica Gel Column Chromatography of the Sulfate Extract From the Pregnancy Urine of Subject TL

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	113-121	190.0	4.38×10^{5}	3.03×10^{4}	14.5
II	122-150	25.7	2.95×10^{4}	3.12×10^{3}	9.5
III	151-160	5.1	2.54×10^{3}	4.00×10^{2}	6.4
IV	161-188	4.3	9.60×10^{3}	1.72×10^{3}	5.6
V	189-258	84.1	1.53×10^{5}	1.52×10^{4}	10.0
VI	259-342	9.8	1.33×10^{5}	1.36×10^{4}	9.8
VII	343-442	42.2	2.76×10^{5}	6.33×10^{4}	4.4
VIII	443-524	32.1	1.03×10^{5}	1.31×10^{4}	7.8
IX	525-560	3.8	1.59×10^{4}	1.84×10^{3}	8.6
X	561-678	64.6	9.30×10^{4}	1.13×10^{4}	8.2
XI	679-709	16.6	1.36×10^{5}	1.99×10^{4}	6.8
XII	710-820	33.1	7.49×10^{4}	2.05×10^{4}	3.6
XIII	821-912	23.3	1.20×10^{5}	1.79×10^{4}	6.7
Strippings		53.5	3.28×10^{4}	6.30×10^{3}	5.2

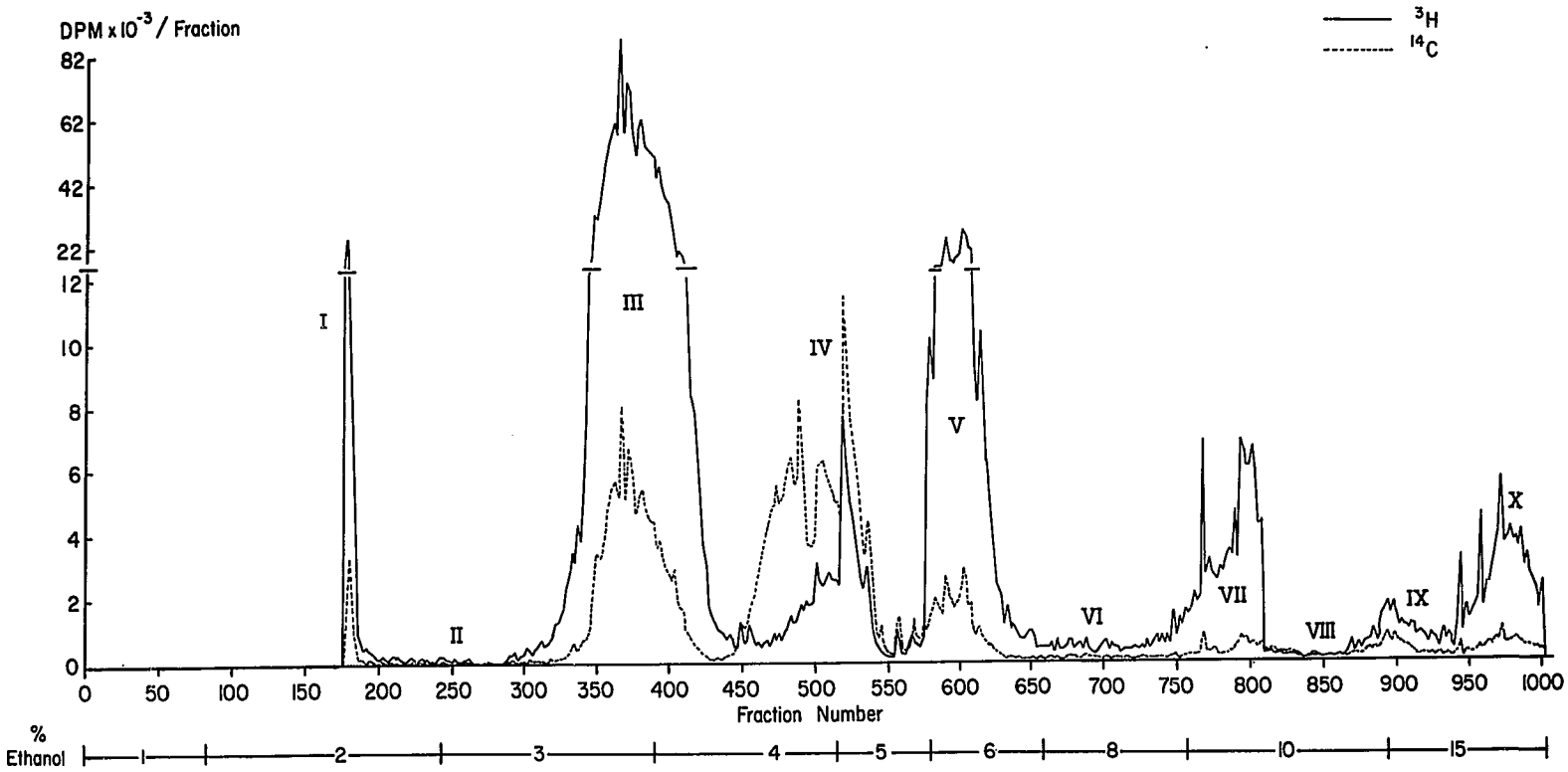


Fig. 24. Silica gel column chromatography of glucosiduronate fraction from pregnancy urine of subject TL.

Table 34

Weight and Radioactivity of Pooled Fractions Obtained After Silica Gel Column Chromatography of the Glucosiduronate Extract From the Pregnancy Urine of Subject TL

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	177-200	280.9	2.18×10^{5}	2.10×10^{4}	10.4
II	201-316	1110.8	5.14×10^{4}	5.92×10^{3}	9.1
III	317-436	72.5	4.06×10^{6}	3.83×10^{5}	10.6
IV	437-552	75.4	4.83×10^{5}	6.11×10^{5}	0.8
V	553-640	217.8	1.09×10^{6}	1.07×10^{5}	10.2
VI	641-746	82.9	6.46×10^{4}	1.02×10^{4}	6.3
VII	747-838	36.7	3.44×10^{5}	3.13×10^{4}	11.0
VIII	839-866	7.0	2.27×10^{4}	3.24×10^{4}	7.0
IX	867-937	31.2	8.87×10^{4}	2.54×10^{4}	3.5
X	937-1000	20.6	2.17×10^{5}	2.44×10^{4}	8.9
Strippings		87.2	8.10×10^{4}	9.00×10^{4}	9.0

2.27×10^6 dpm of ^3H and 2.10×10^5 dpm of ^{14}C. Half of this residue was combined with 41.20 mg of carrier 15α-hydroxyandrostenedione and the mixture was crystallized to constant specific activity as shown in Table 35. The final crystals, which weighed 20.0 mg, were reduced with $NaBH_4$, oxidized with DDQ and acetylated in the manner described earlier. The product obtained was chromatographed on a 3 g alumina column, and the residue eluted with 60% benzene in hexane weighed 11.65 mg and contained 2.11×10^5 dpm of H^3 and 1.46×10^4 dpm of ^{14}C. Two crystallizations of this material from ethanol showed that it was radiochemically pure (Table 35). The infrared spectrum (KBr) of an aliquot of the second crystals was identical to that of authentic 15α-hydroxytestosterone diacetate.

The residue obtained from Pool IV (Figure 24, Table 34) was chromatographed on a 50 g Celite column using system D. Five-ml fractions were collected and the distribution of radioactivity from the column is shown in Figure 25. The weights and dpm of the pooled fractions is given in Table 36.

Table 36

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Residue From Pool IV (Figure 24, Table 34)

Pool	Fraction No.	Weight (mg)	^3H (dpm)	^{14}C (dpm)	$^3H/^{14}C$
I	44-57	4.4	2.66×10^5	3.31×10^4	8.0
II	58-108	5.8	3.57×10^4	4.43×10^5	0.1
Strippings		31.9	1.97×10^4	1.06×10^4	1.8

Table 35

Proof of Radiochemical Purity of 15α-Hydroxyandrostenedione Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject TL

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyandrostenedione						
1	25,270	2,330	10.8	35,310	3,260	10.8
2	24,320	2,240	10.8	27,480	2,550	10.9
3	24,820	2,340	10.6	24,910	2,310	10.8
Calculated	27,600*	2,550*				
15α-Hydroxytestosterone diacetate						
1	20,120	1,860	10.8	14,060	1,300	10.8
2	20,560	1,920	10.7	20,490	1,880	10.9
Calculated	21,470**	1,820**				

*A total of 1,137,000 dpm of ^{3}H and 105,100 dpm of ^{14}C was mixed with 41.20 mg of carrier 15α-hydroxyandrostenedione prior to crystallization.

**This value was computed by using the final specific activity of the crystals of 15α-hydroxyandrostenedione and adjusting for the change in molecular weight.

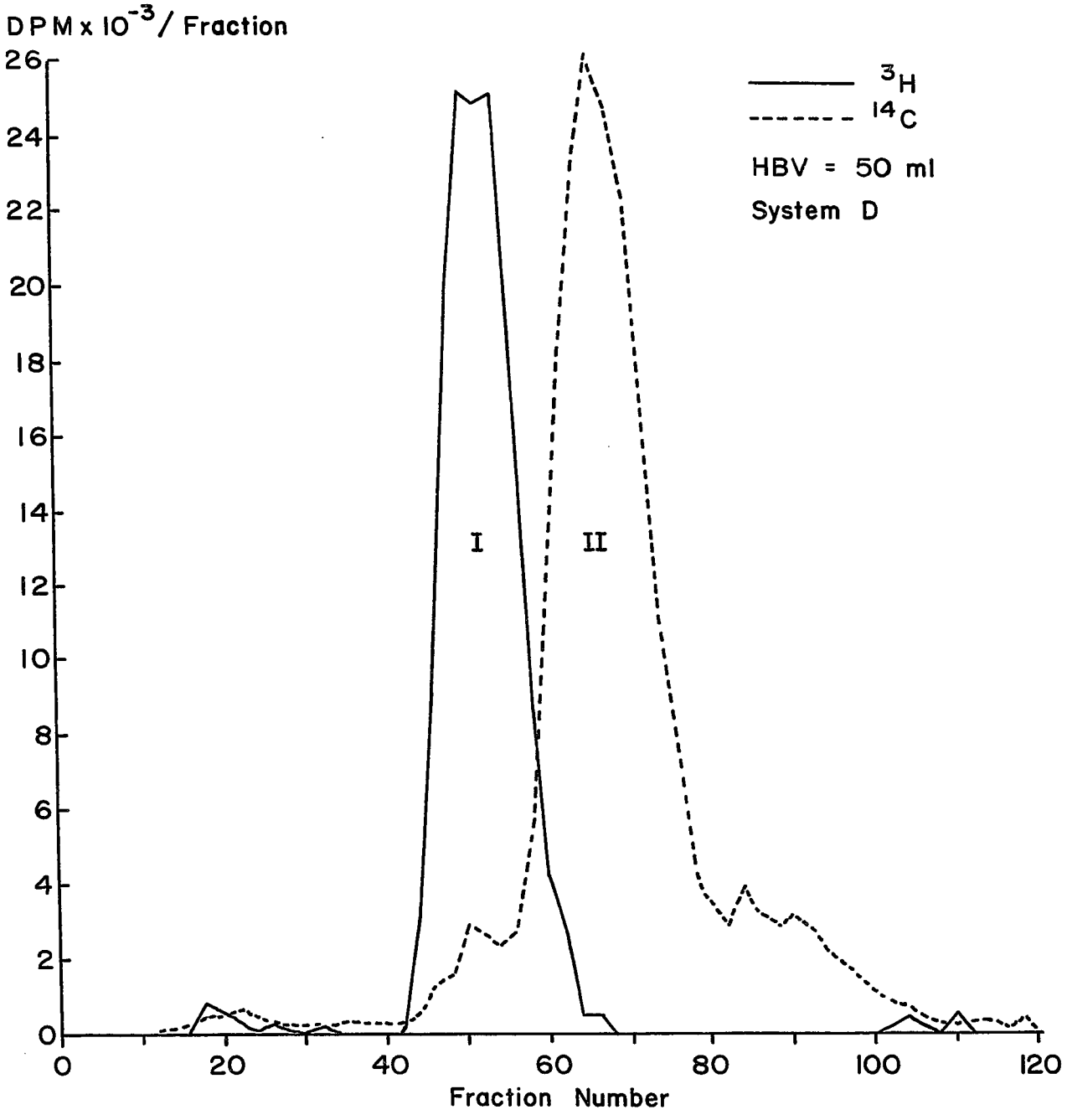


Fig. 25. Celite column chromatography of residue of Pool IV (Fig. 24, Table 34).

The material from Pool I (Figure 25, Table 36) was purified directly by paper chromatography in system O for 43 hours, however, it did not migrate with the mobility of any of the available standard 15α-hydroxylated steroids and, as a result, it could not be identified.

The residue from Pool II (Figure 25, Table 36) was chromatographed on paper in system M for 3 1/2 hours and a single peak of radioactivity, which migrated close to authentic 15α-hydroxydehydroisoandrosterone, run alongside, was detected on the chromatogram. The material eluted from the radioactive zone was then chromatographed in system O for 50 1/2 hours and resolved into two areas of radioactive material. The less polar material migrated approximately 4 cm ahead of standard 15α-hydroxydehydroisoandrosterone and, on elution, yielded an oily residue which weighed 0.5 mg and contained 3.66×10^4 dpm of ^3H and 4.05×10^5 dpm of ^{14}C. Attempts to crystallize this material were unsuccessful and it was not processed further. The more polar zone had the same mobility as 15α-hydroxydehydroisoandrosterone and elution of the material afforded 0.2 mg of residue which contained 3.90×10^3 dpm of ^3H and 4.43×10^4 dpm of ^{14}C. Half of this material was mixed with 14.05 mg of carrier 15α-hydroxydehydroisoandrosterone and the mixture was crystallized to constant specific activity as shown in Table 37. All of the tritium in the mixture partitioned into the mother liquor.

Chromatography of the residue of Pool V (Figure 24, Table 34) on a 97 g Celite column (HBV = 190 ml) in system E yielded a single peak of radioactivity. The material comprising this peak weighed 20.7 mg and had 9.58×10^5 dpm of ^3H and 9.83×10^4 dpm of ^{14}C. It was further purified by paper chromatography in system M for 3 1/2 hours and migrated close to

15α-hydroxyestrone, run as a standard. This material was not identified since it could not be matched with any of the available authentic 15α-hydroxylated steroids.

The residue from Pool VII (Figure 24, Table 34), was purified directly by paper chromatography in system M for 48 hours and it resolved into two zones of radioactive material. The more polar of the two zones contained 6.90×10^4 dpm of ^3H and $6.45 \times {}^4 10$ dpm of 10^4, and migrated close to the origin, whereas the less polar zone had the same mobility as 15α-hydroxyestradiol. A third zone of radioactivity (1.40×10^5 dpm of ^3H and 9.30×10^4 dpm of ^{14}C) was in the runoff. The material eluted from the less polar zone weighed 1.30 mg and had 3.21×10^4 dpm of ^3H and 2.60×10^4 dpm of ^{14}C. It was mixed with 9.50 mg of carrier 15α-hydroxyestradiol and was crystallized to constant $^3H/^{14}C$ ratio as shown in Table 38. The final crystals and mother liquor were combined and gave a weight of 4.3 mg. This material was acetylated and the product was crystallized to constant specific activity as shown in Table 38. The infrared spectrum (KBr) of an aliquot of the second crystals matched that of authentic 15α-hydroxyestradiol triacetate.

The material from Pool IX (Figure 24, Table 24) was chromatographed on paper in system W for 4 hours, yielding two peaks of radioactive material. The less polar material migrated close to the standard 15α-hydroxyestradiol, run alongside, and could not be identified, whereas the more polar material had the same mobility as 15α-hydroxyestriol. The eluted material from the more polar zone weighed 11.1 mg and contained 3.59×10^4 dpm of ^3H as well as 6.00×10^4 dpm of ^{14}C. It was further purified in system X for 24 1/2 hours and, on elution of the radioactive

material corresponding in mobility to 15α-hydroxyestriol yielded 3.37 x 10^4 dpm of ^3H and 4.85 x 10^4 of ^{14}C and weighed 1.3 mg. This residue was mixed with 10.50 mg of carrier 15α-hydroxyestriol and the mixture was crystallized as shown in Table 39. A total of 5.1 mg of the last crystals and mother liquor was acetylated and the acetylated product was crystallized to constant specific activity (Table 39). The infrared spectrum (KBr) of the second crystals was identical to that of authentic 15α-hydroxyestriol tetraacetate.

Table 37

Proof of Radiochemical Purity of 15α-Hydroxydehydroisoandrosterone Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject TL

	Specific Activity (dpm ^{14}C/mg)	
Crystallization	Crystals	Mother Liquor
1	3060	2560
2	3080	2660
3	3120	2950
Calculated	3110*	

*A total of 43,650 dpm of ^{14}C was mixed with 14.05 mg of carrier 15α-hydroxydehydroisoandrosterone prior to crystallization.

Table 38

Proof of Radiochemical Purity of 15α-Hydroxyestradiol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject TL

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestradiol						
1	2590	170	15.3	2520	260	9.8
2	2590	170	15.4	2530	180	14.0
3	2600	170	15.7	2630	170	15.1
Calculated	3380*	270*				
15α-Hydroxyestradiol triacetate						
1	1900	120	15.8	1580	150	10.5
2	1880	120	15.7	1860	120	15.5
Calculated	1810**	120**				

*A total of 32,100 dpm of ^{3}H and 2,600 dpm of ^{14}C was mixed with 9.50 mg of carrier 15α-hydroxyestradiol prior to crystallization.

**This value was computed by using the final specific activity of the crystals of 15α-hydroxyestradiol and adjusting for the change in molecular weight.

Table 39

Proof of Radiochemical Purity of 15α-Hydroxyestriol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject TL

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestriol						
1	1650	160	10.3	4420	770	5.7
2	1590	150	10.6	2010	240	8.4
3	1610	150	10.7	1660	160	10.4
Calculated	3050*	450*				
15α-Hydroxyestriol tetraacetate						
1	1130	110	10.3	1100	110	10.0
2	1130	110	10.3	1120	100	11.2
Calculated	1040**	100**				

*A total of 32,000 dpm of ^{3}H and 4,700 dpm of ^{14}C was mixed with 10.5 mg of carrier 15α-hydroxyestriol prior to crystallization.

**This value was obtained by using the final specific activity of the crystals of 15α-hydroxyestriol and adjusting for the change in molecular weight.

II. Subject MP

In order to confirm the results just described, a 21-year old subject (MP) in the 30th week of pregnancy was given an intravenous injection of 1.61×10^7 dpm of ^{3}H-15α-hydroxyandrostenedione (specific activity 1.66×10^6 dpm/μg) and 3.13×10^6 dpm of ^{14}C-15α-hydroxydehydroisoandrosterone (specific activity 4.93×10^4 dpm/μg), $^3H/^{14}C = 5.1$. Urine was collected for four days and the amount of radioactivity measured in each day's urine is shown in Table 40. The urine was then pooled and the conjugates were hydrolyzed as in the previous experiment; the weight and radioactivity of the sulfate and glucosiduronate extracts are given in Table 41.

Table 40

Radioactivity Excreted in the Urine Following the Intravenous Injection of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone in Subject MP

Day	Volume (ml)	^{3}H (dpm)	% of I.D.*	^{14}C (dpm)	% of I.D.	$^3H/^{14}C$
1	980	1.07×10^7	66.4	1.34×10^6	42.8	8.0
2	760	4.29×10^5	2.7	2.39×10^4	0.7	17.9
3	940	2.43×10^5	1.5	1.27×10^4	0.4	19.1
4	900	1.42×10^5	0.8	8.10×10^3	0.2	17.5
TOTAL	3580	1.15×10^7	71.4	1.38×10^6	44.1	8.3

*I.D. = Injected Dose

Table 41

Weight and Radioactivity of the Conjugated Fractions From the Pregnancy Urine of Subject MP

Fraction	Weight (g)	^{3}H (dpm)	% of dpm in Urine	^{14}C (dpm)	% of dpm in Urine	$^{3}H/^{14}C$
Sulfate	0.77	1.06×10^{6}	9.2	9.08×10^{4}	6.6	11.7
Glucosiduronate	0.80	7.55×10^{6}	65.6	1.27×10^{6}	92.0	5.9
TOTAL		8.61×10^{6}	74.8	1.36×10^{6}	98.6	6.3

Purification of the glucosiduronate extract was achieved by first chromatographing this fraction on a 350 g silica gel column using increasing concentrations of ethanol in methylene dichloride. A graph of the distribution of radioactivity eluted from the column is presented in Figure 26, and Table 42 shows the weight and radioactivity of the pooled fractions from this column.

Table 42

Weight and Radioactivity of Pooled Fractions Obtained After Silica Gel Column Chromatography of the Glucosiduronate Extract From the Pregnancy Urine of Subject MP

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	238-279	284.0	3.12×10^{5}	3.76×10^{4}	8.3
II	280-422	284.2	6.70×10^{4}	1.04×10^{4}	6.4
III	423-552	100.8	3.52×10^{5}	2.42×10^{5}	14.5
IV	553-732	96.8	3.11×10^{5}	6.01×10^{5}	0.5
V	733-906	103.7	8.05×10^{5}	7.23×10^{4}	11.1
VI	907-1031	91.0	7.44×10^{4}	9.95×10^{3}	7.5
VII	1032-1191	78.1	2.78×10^{5}	2.36×10^{4}	11.7
VIII	1192-1341	50.2	7.11×10^{4}	2.91×10^{4}	2.4
Strippings		139.0	1.88×10^{5}	2.07×10^{4}	9.1

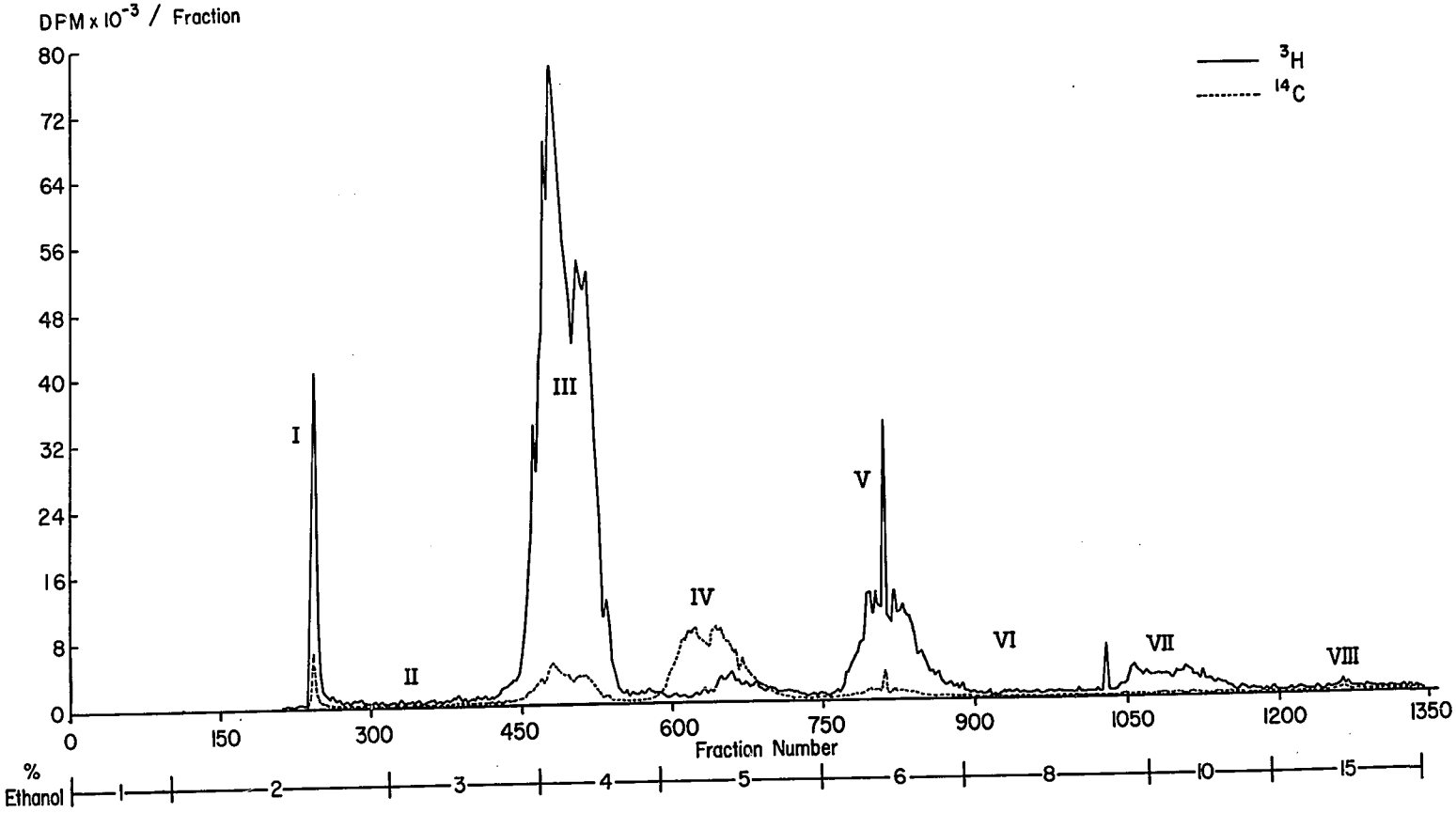


Fig. 26. Silica gel column chromatography of glucosiduronate fraction from pregnancy urine of subject MP.

The material in Pool III (Figure 26, Table 42) was further purified by chromatography in system D using 100 g of Celite (HBV = 200 ml). Ten-ml fractions were collected and a single peak of radioactive material was eluted in the fourth and fifth hold-back volumes. The residue from this peak had a weight of 6.7 mg and contained 3.22×10^6 dpm of ^3H and 2.05×10^5 dpm of ^{14}C. It was then purified by paper chromatography in systems O (19 hours) and M (4 hours). In both systems a single peak of radioactive material, which corresponded in mobility to 15α-hydroxyandrostenedione was detected. Elution of the radioactive material from the last chromatogram yielded a residue with 2.80×10^6 dpm of ^3H and 1.79×10^5 dpm of ^{14}C and a weight of 2.7 mg. Half of this material was mixed with 45.65 mg of carrier 15α-hydroxyandrostenedione and crystallized as shown in Table 43. The last crystals which weighed 28.0 mg were acetylated, reduced with $NaBH_4$ and oxidized with the DDQ reagent, and the product formed was chromatographed on a 6 g alumina column. Elution with 0.5% ethanol in benzene gave a residue, which had a weight of 22.9 mg and 5.09×10^5 dpm of ^3H as well as 2.04×10^4 dpm of ^{14}C. This material was mixed with 45.65 mg of carrier 15α-hydroxyandrostenedione and, after three crystallizations, its radiochemical homogeneity was established (Table 43).

Pool IV (Figure 26, Table 42) was chromatographed on a 75 g Celite column using system D, and 10 ml fractions were collected. The distribution of radioactivity obtained in the eluted fractions is shown in Figure 27. Table 44 gives the weight and dpm of the pooled fractions off the column.

Table 43

Proof of Radiochemical Purity of 15α-Hydroxyandrostenedione Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject MP

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyandrostenedione						
1	30,010	1,820	16.5	28,890	1,810	15.9
2	30,640	1,880	16.3	30,280	1,840	16.4
3	29,370	1,830	16.1	29,110	1,810	16.1
Calculated	31,040*	1,940*				
15α-Acetoxytestosterone						
1	26,740	1,640	16.3	15,850	980	16.2
2	28,140	1,740	16.2	26,230	1,580	16.5
3	28,540	1,720	16.5	28,270	1,730	16.4
Calculated	26,640**	1,600**				

*A total of 1,432,900 dpm of ^{3}H and 100,900 dpm of ^{14}C was mixed with 45.65 mg of carrier 15α-hydroxyandrostenedione prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 15α-hydroxyandrostenedione and adjusting for the change in molecular weight.

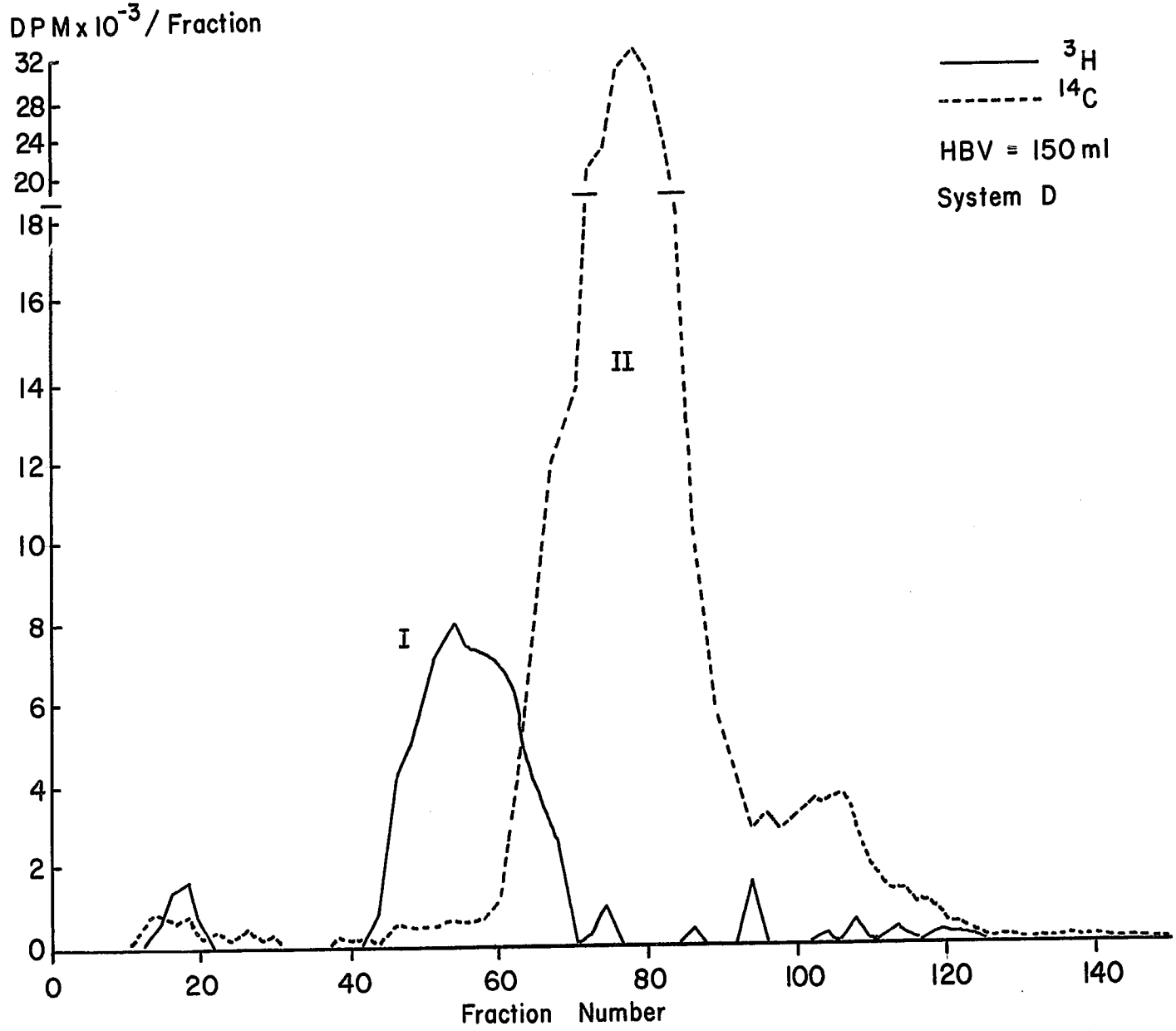


Fig. 27. Celite column chromatography of the residue of Pool IV (Fig.26, Table 42).

Table 44

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Residue From Pool IV (Figure 26, Table 42)

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	44-62	4.1	1.09×10^{5}	1.21×10^{4}	9.0
II	63-124	6.5	5.92×10^{4}	5.71×10^{5}	0.1
Strippings		36.4	3.30×10^{4}	1.55×10^{4}	2.1

The material of Pool II (Figure 27, Table 44) was purified by paper chromatography in system O for 40 hours and resolved into two peaks of radioactive material. The less polar material which migrated ahead (approximately 4 cm) of 15α-hydroxydehydroisoandrosterone, run as a standard, yielded 3.20×10^{3} dpm of ^{3}H and 4.47×10^{5} dpm of ^{14}C on elution. This material was not processed further. The more polar zone had the same mobility as authentic 15α-hydroxydehydroisoandrosterone, run alongside, and the residue from the elution of the radioactive material was chromatographed in system M for 4 hours. A single radioactive peak of material was detected on the chromatogram and gave 1.20×10^{3} dpm of ^{3}H and 9.51×10^{4} dpm of ^{14}C on elution. This residue, which weighed 0.45 mg, was combined with 9.15 mg of carrier 15α-hydroxydehydroisoandrosterone and, after four crystallizations of this mixture, constant specific activity was attained as shown in Table 45. All of the tritium was found in the first mother liquor.

Table 45

Proof of Radiochemical Purity of 15α-Hydroxydehydroisoandrosterone Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject MP

	Specific Activity (dpm ^{14}C/mg)	
Crystallization	Crystals	Mother Liquor
1	9,580	4,740
2	9,510	6,720
3	9,760	8,130
4	9,990	9,610
Calculated	10,390*	

*A total of 95,070 dpm of ^{14}C was mixed with 9.15 mg of carrier 15α-hydroxydehydroisoandrosterone prior to crystallization.

The residue of Pool VII (Figure 26, Table 42) was chromatographed on a 50 g Celite column using system F. A plot of the radioactivity eluted versus fraction number is shown in Figure 28, and Table 46 gives the weight and radioactivity of the pooled fractions.

Table 46

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Residue of Pool VII (Figure 26, Table 42)

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	12-33	25.5	3.68×10^{4}	4.56×10^{3}	8.1
II	34-84	12.1	2.21×10^{5}	1.00×10^{4}	22.1
Strippings		20.6	1.05×10^{4}	1.50×10^{3}	7.0

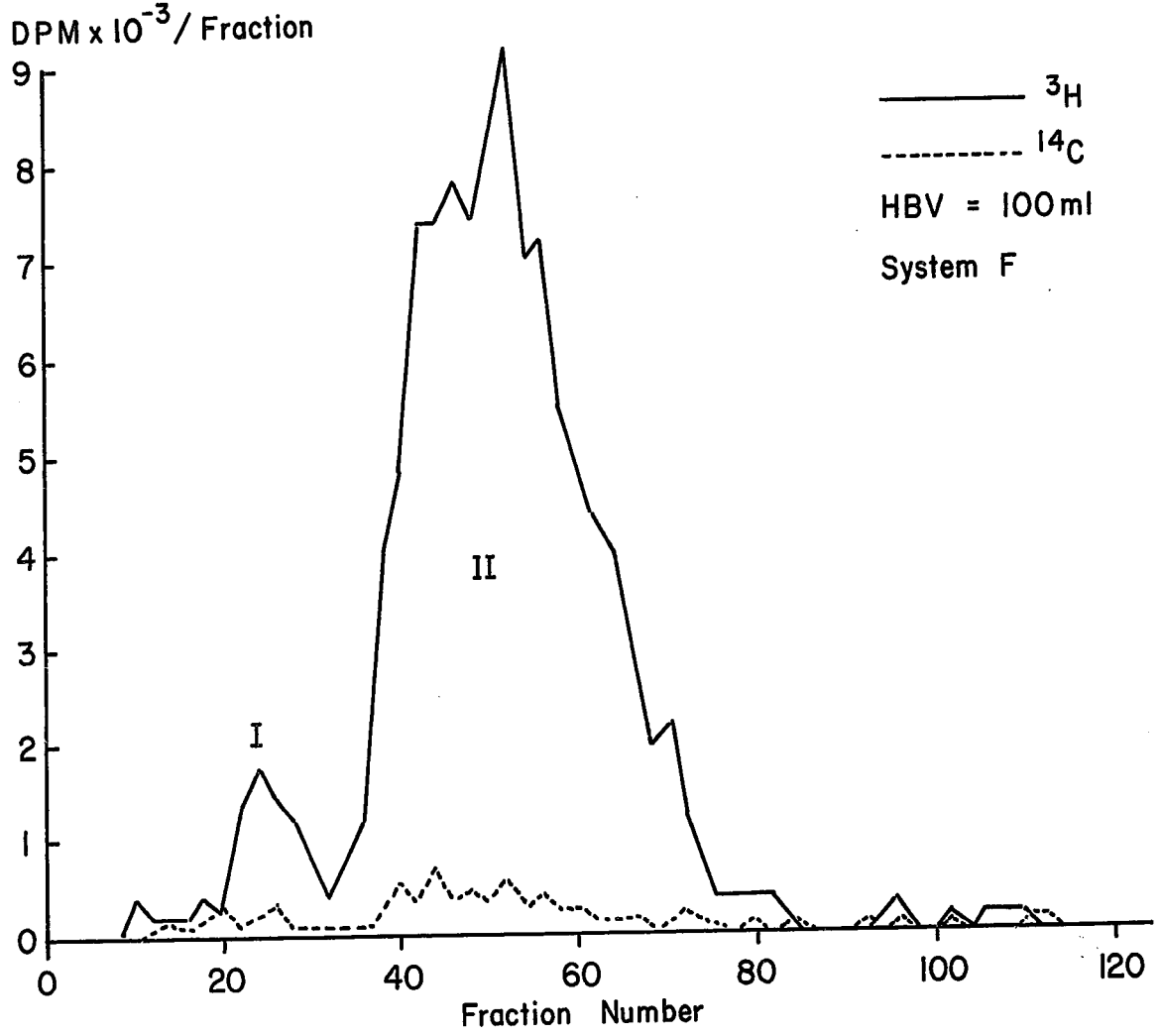


Fig. 28. Celite column chromatography of residue of Pool VII (Fig.26, Table 42)

Chromatography of the residue of Pool II (Figure 28, Table 46) on paper in system X for 24 hours yielded two peaks of radioactive material. The less polar material (zone A) migrated with the same mobility as authentic 15α-hydroxyestradiol, whereas the more polar material (zone B) had the same mobility as 15α-hydroxyestriol. The residue from the elution of zone A weighed 1.0 mg and contained 2.95×10^4 dpm of ^3H and 2.25×10^3 dpm of ^{14}C. It was mixed with 9.60 mg of carrier 15α-hydroxyestradiol and the mixture was crystallized. After three crystallizations, the specific activities of the crystals and mother liquor were not constant as shown in Table 47. A derivative was prepared by acetylation of the second and third crystals, as well as the final mother liquor (4.2 mg), and the product obtained was crystallized to constant specific activity as shown in Table 47.

The residue obtained after elution of the material in zone B of the last chromatogram was further purified by paper chromatography in system W for 4 hours. A single radioactive zone was detected and on elution gave 4.53×10^4 dpm of ^3H and 2.85×10^3 of ^{14}C. This residue (1.3 mg) was combined with 10.30 mg of carrier 15α-hydroxyestriol and was crystallized to constant $^3H/^{14}C$ ratio (Table 48). Acetylation of the last crystals and mother liquor (5.1 mg) yielded a product which, after three crystallizations, was found to be radiochemically pure (Table 48).

Table 47

Proof of Radiochemical Purity of 15α-Hydroxyestradiol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject MP

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestradiol						
1	1000	45	22.2	6050	479	13.6
2	950	35	27.1	2290	162	14.1
3	920	36	25.5	1110	54	20.5
Calculated	3070*	230*				
15α-Hydroxyestradiol triacetate						
1	820	34	24.1	960	161	6.0
2	820	34	24.1	790	47	16.8
3	860	36	23.9	880	36	24.4
Calculated	960**	42**				

*A total of 29,500 dpm of ^{3}H and 2,250 dpm of ^{14}C was mixed with 9.60 mg of carrier 15α-hydroxyestradiol prior to crystallization.

**This value was calculated by using the final specific activities of the second and third crystals and the final mother liquor of 15α-hydroxyestradiol, and adjusting for the change in molecular weight.

Table 48

Proof of Radiochemical Purity of 15α-Hydroxyestriol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject MP

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestriol						
1	3630	220	16.7	3730	260	14.5
2	3620	220	16.6	3390	210	16.0
3	3670	230	16.2	3540	220	16.4
Calculated	4530*	200*				
15α-Hydroxyestriol tetraacetate						
1	2020	120	16.8	1950	120	16.2
2	2010	120	16.7	2060	120	17.2
3	2650	160	16.6	2650	160	16.6
Calculated	2360**	150**				

*A total of 46,700 dpm of ^{3}H and 3,050 dpm of ^{14}C was mixed with 10.30 mg of carrier 15α-hydroxyestriol prior to crystallization.

**This value was computed by using the final specific activity of the crystals of 15α-hydroxyestriol and adjusting for the change in molecular weight.

Experiment 5 - Metabolism of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone Injected Into the Fetus in Utero

In the studies to be described in this section, a mixture of labeled 15α-hydroxyandrostenedione and 15α-hydroxydehydroisoandrosterone was injected into the peritoneal cavity of two fetuses at the time of intrauterine transfusion for erythroblastosis fetalis as described earlier. The maternal urine was then collected for five days and was analyzed.

I. Subject BB

This woman was in the 29th week of gestation and was undergoing her third IUT. There were no fetal ascites at the first or second IUT, but there was some fluid and clotted blood in the fetal peritoneum at the third IUT. On discharge, the fetus was alive as judged by the fetal heart recordings. The urine collection on the second day after the injection was very large because the subject was placed on a diuretic. Also, approximately 8 ml of blood was lost due to a syringe breaking.

Subject BB was injected with a mixture of 1.33×10^{7} dpm of ^{3}H-15α-hydroxyandrostenedione (specific activity 1.66×10^{6} dpm/μg) and 2.85×10^{6} dpm of ^{14}C-15α-hydroxydehydroisoandrosterone (specific activity 4.93×10^{4} dpm/μg), $^{3}H/^{14}C = 4.7$. Table 49 gives the volume of urine collected each day and the radioactivity recovered in this urine. After pooling the urine, the unconjugated and conjugated steroids were extracted and the weight and radioactivity of these extracts are presented in Table 50.

Table 49

Radioactivity Excreted in the Urine Following the Administration of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone in Subject BB

Day	Volume (ml)	^{3}H (dpm)	% of I.D.*	^{14}C (dpm)	% of I.D.	^{3}H/^{14}C
1	1000	3.55×10^{6}	26.7	5.53×10^{5}	19.4	6.4
2	4260	1.11×10^{6}	8.3	1.83×10^{5}	6.4	6.1
3	1000	3.10×10^{5}	2.3	2.20×10^{4}	0.8	14.1
4	750	1.57×10^{5}	1.2	9.75×10^{3}	0.3	16.1
5	700	6.54×10^{4}	0.5	4.55×10^{3}	0.1	14.4
TOTAL	7710	5.19×10^{6}	39.0	7.72×10^{5}	27.0	6.7

*I.D. = Injected Dose

Table 50

Weight and Radioactivity of the Unconjugated and Conjugated Fractions From the Pregnancy Urine of Subject BB

Fraction	Weight (g)	^{3}H (dpm)	% of dpm in Urine	^{14}C (dpm)	% of dpm in Urine	^{3}H/^{14}C
Unconjugated	0.9	2.44×10^{4}	0.5	2.90×10^{3}	0.4	8.4
Sulfate	3.2	4.06×10^{5}	8.0	6.22×10^{4}	8.0	6.5
Glucosiduronate	2.1	1.71×10^{6}	32.9	2.89×10^{5}	37.4	5.9
TOTAL		2.14×10^{6}	41.4	3.54×10^{5}	45.8	6.0

The sulfate fraction (Table 50) was purified on a 225 g silica gel column using increasing concentrations of ethanol in methylene dichloride. A plot of the radioactivity recovered against the fraction number is shown in Figure 29. The weight and radioactivity obtained after pooling the fractions is shown in Table 51. These fractions contained small amounts of radioactivity and were very impure. Only the material from Pools III and V was processed further, but could not be identified after purification by paper chromatography.

Table 51

Weight and Radioactivity of Pooled Fractions Obtained After Silica Gel Column Chromatography of the Sulfate Fraction From the Pregnancy Urine of Subject BB

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	164-173	317.2	2.73×10^{4}	5.10×10^{3}	5.3
II	174-250	88.4	2.37×10^{4}	1.24×10^{4}	1.9
III	251-301	61.4	9.43×10^{4}	5.85×10^{3}	16.1
IV	302-478	228.2	1.01×10^{5}	1.69×10^{4}	5.9
V	479-539	49.3	9.48×10^{4}	9.10×10^{3}	10.4
VI	540-578	34.0	3.69×10^{4}	4.90×10^{3}	9.7
Strippings		393.3	2.88×10^{4}	7.95×10^{3}	3.6

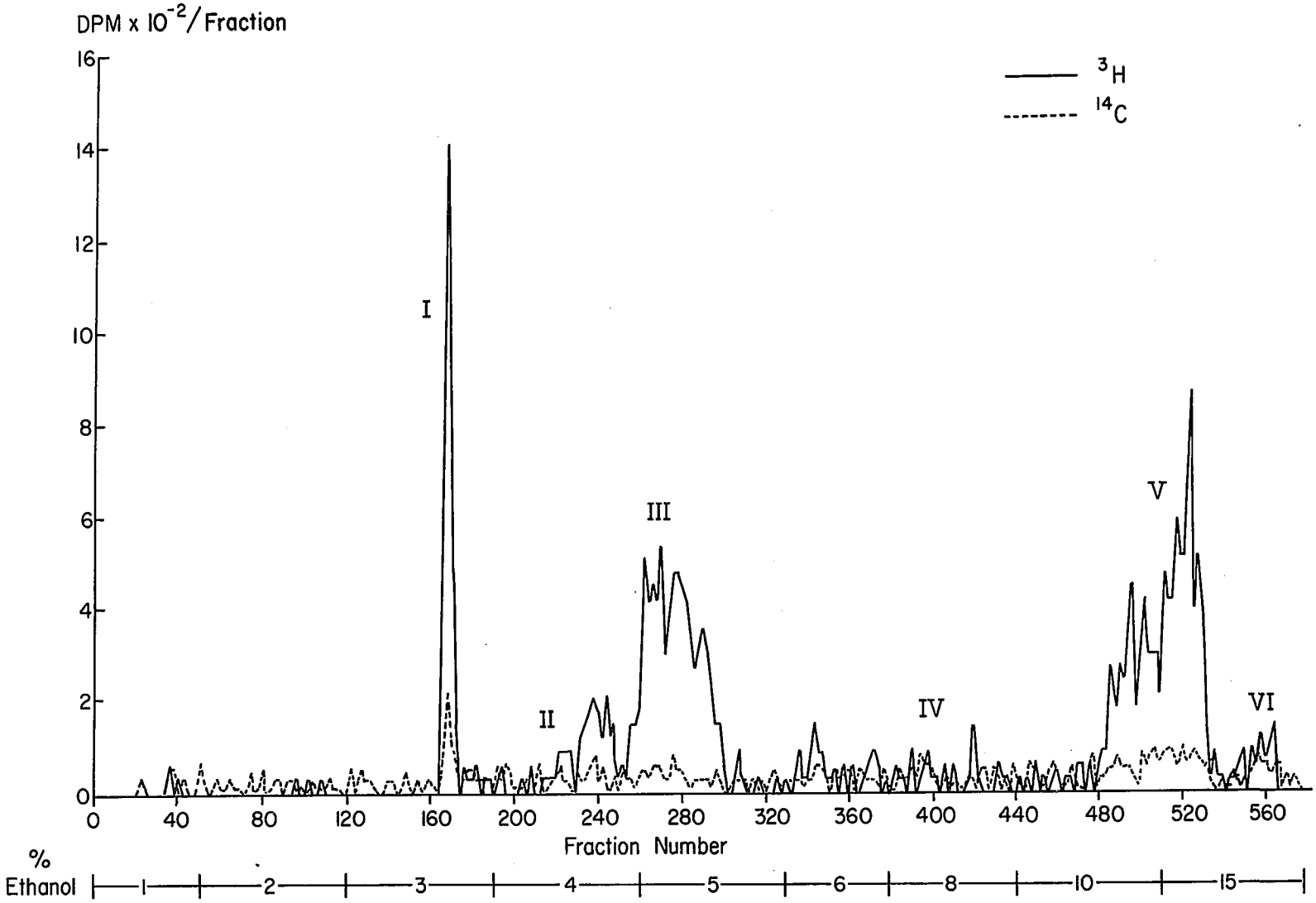


Fig. 29. Silica gel column chromatography of sulfate fraction from pregnancy urine of subject BB.

The glucosiduronate extract was also purified by silica gel column chromatography. It was applied on a 250 g silica gel column and fractions were eluted with increasing concentrations of ethanol in methylene dichloride. The distribution of radioactive material eluted from the column is shown graphically in Figure 30 and the weight and dpm of the material from the various pools is given in Table 52. The material from Pools III, IV and VIII was processed, whereas the residues from the other pools are now being purified.

Table 52

Weight and Radioactivity of Pooled Fractions Obtained After Silica Gel Column Chromatography of the Glucosiduronate Fraction From the Pregnancy Urine of Subject BB

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	152-176	280.7	3.50×10^{4}	7.75×10^{3}	4.5
II	177-300	465.9	2.50×10^{4}	6.30×10^{3}	4.0
III	*	135.0	3.20×10^{5}	3.47×10^{4}	9.2
IV	1302-1414	258.3	1.78×10^{5}	1.53×10^{5}	1.1
V	1415-1512	180.0	8.52×10^{4}	3.20×10^{4}	2.7
VI	1513-1546	52.6	2.31×10^{4}	6.10×10^{3}	3.8
VII	1547-1600	38.6	1.24×10^{5}	1.54×10^{4}	8.0
VIII	1601-1666	78.2	1.05×10^{5}	2.23×10^{4}	4.7
Strippings		138.9	4.56×10^{4}	1.21×10^{4}	3.8

*This pool was recovered from the turntable after it had unexpectedly stopped rotating.

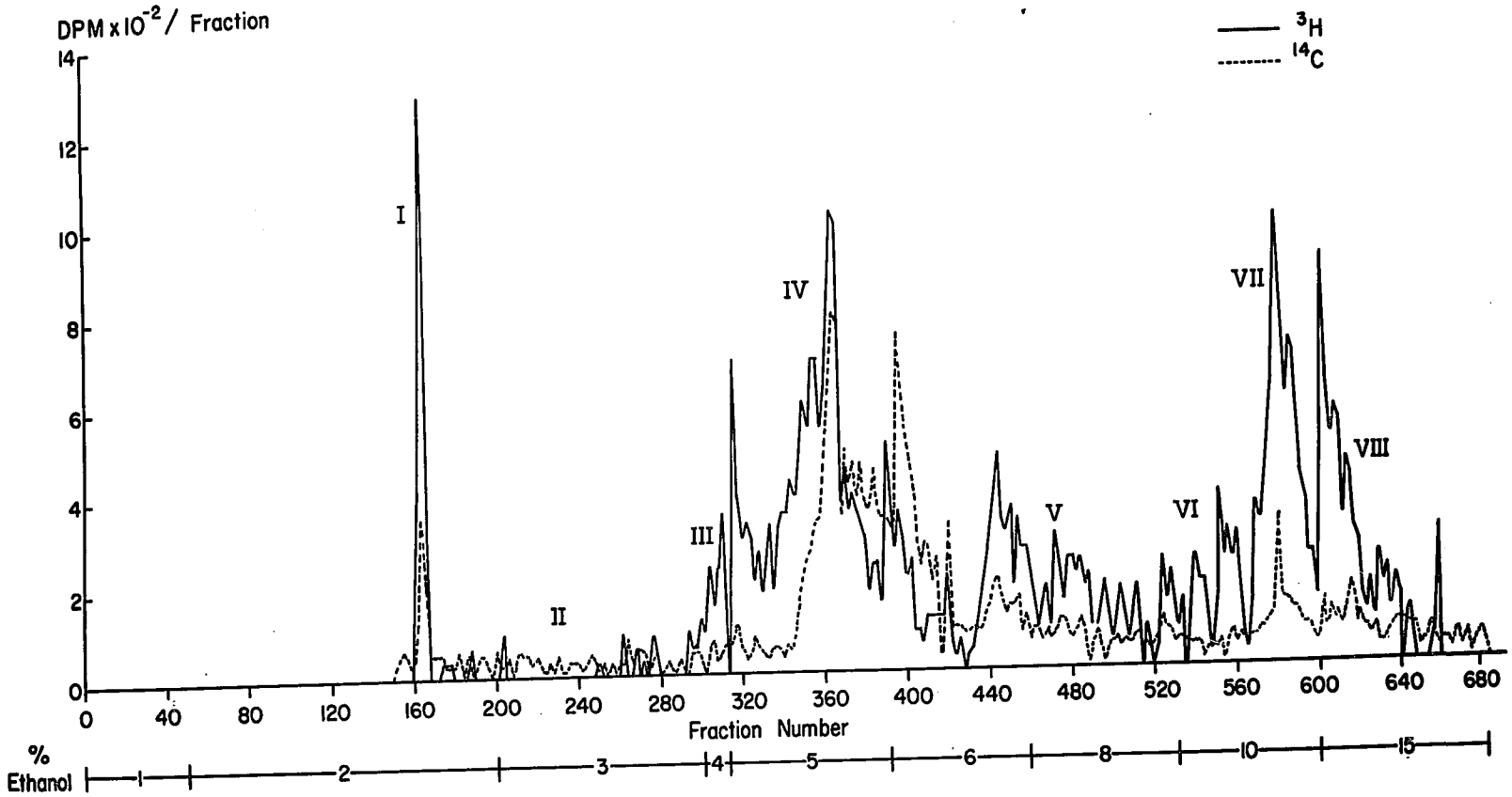


Fig. 30. Silica gel column chromatography of glucosiduronate fraction from pregnancy urine of subject BB.

The residue from Pool III (Figure 30, Table 52) was further purified by Celite column (75 g) chromatography in system D. Ten-ml fractions were collected, and only one peak of radioactive material was eluted in the third to fifth hold-back volumes. This material weighed 7.9 mg and contained 2.43×10^5 dpm of ^3H as well as 3.06×10^4 dpm of ^{14}C. It was purified by paper chromatography sequentially in systems O (24 1/4 hours) and M (4 hours) and in both systems a single band of radioactive material with the mobility of authentic 15α-hydroxyandrostenedione was detected on scanning. The eluted material contained 1.84×10^5 dpm of ^3H and 2.22×10^4 dpm of ^{14}C and weighed 0.6 mg. Half of this residue was then mixed with 24.90 mg of carrier 15α-hydroxyandrostenedione. After three crystallizations, the $^3H/^{14}C$ ratio of the crystals and mother liquor was constant as shown in Table 53. The last crystals (12.0 mg) were then reduced with $NaBH_4$, oxidized with the reagent DDQ and acetylated as described previously. The resulting product was purified on a 2 g alumina column and elution with 60% benzene in hexane yielded a residue which contained 2.58×10^4 dpm of ^3H and 2.40×10^3 dpm of ^{14}C and weighed 5.5 mg. Two crystallizations of this material showed that it was radiochemically pure (Table 53). Infrared analysis (KBr) gave the same spectrum as that of 15α-hydroxytestosterone diacetate.

The material in Pool IV (Figure 30, Table 52) was chromatographed on a 100 g Celite column using system D. Ten-ml fractions were collected and two radioactive peaks were recovered from the column as shown in Figure 31. The weight and radioactivity of the material obtained after pooling the fractions in each peak are given in Table 54.

Table 53

Proof of Radiochemical Purity of 15α-Hydroxyandrostenedione Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject BB

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyandrostenedione						
1	7050	880	8.0	7950	970	8.2
2	7010	860	8.2	7500	890	8.4
3	6980	840	8.3	7080	860	8.3
Calculated	7400*	890*				
15α-Hydroxytestosterone diacetate						
1	5560	680	8.2	3420	410	8.3
2	5760	700	8.2	5770	690	8.4
Calculated	5460**	660**				

*A total of 184,615 dpm of ^{3}H and 22,251 dpm of ^{14}C was mixed with 24.95 mg of carrier 15α-hydroxyandrostenedione prior to crystallization.

**This value was computed by using the specific activity of the last crystals of 15α-hydroxyandrostenedione and adjusting for the change in molecular weight.

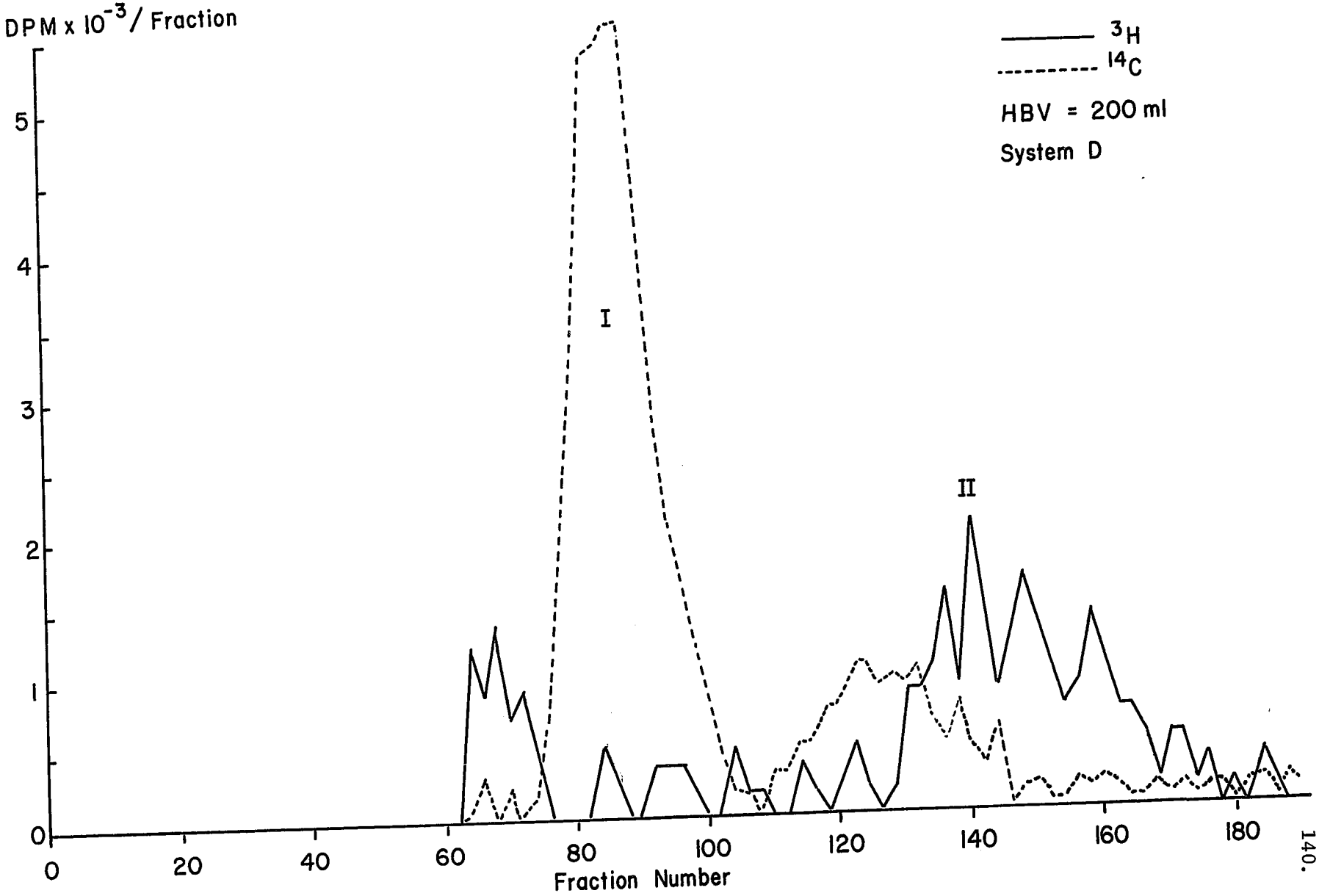


Fig. 31. Celite column chromatography of residue of Pool IV (Fig. 30, Table 52).

Table 54

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Residue of Pool IV (Figure 30, Table 54)

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	74-106	6.3	7.30×10^{3}	8.18×10^{4}	0.1
II	107-193	11.2	6.80×10^{4}	2.92×10^{4}	2.3
Strippings		97.7	3.54×10^{4}	1.39×10^{4}	2.5

The residue from Pool I (Figure 31, Table 54) was purified by paper chromatography in system O for 30 hours and a single zone of radioactive material, which migrated approximately 5 cm ahead of 15α-hydroxydehydroisoandrosterone, run as a standard, was detected. The material eluted contained 3.40×10^{4} dpm of ^{3}H and 6.98×10^{4} dpm of ^{14}C. Nothing further was done with this material, as it could not be matched in mobility to an authentic standard. The area on the chromatogram corresponding in mobility to 15α-hydroxydehydroisoandrosterone was also processed but no radioactivity was eluted. The material in Pool II (Figure 31, Table 54) was also purified by paper chromatography but could not be identified.

Purification of the material in Pool VII (Figure 30, Table 52) by paper chromatography in system W for 4 hours, yielded two radioactive bands. The more polar band migrated close to standard 15α-hydroxyestriol and was not processed further, whereas the less polar band had the same mobility as authentic 15α-hydroxyestradiol. The material obtained from the latter zone was chromatographed further in system M for 52 hours. On elution, a residue, which contained 2.55×10^{4} dpm of ^{3}H and 2.30×10^{3} dpm of ^{14}C and weighed 1.05 mg, was obtained. It was combined with

9.45 mg of carrier 15α-hydroxyestradiol and the mixture was crystallized to constant specific activity as shown in Table 55. A derivative was formed by combining the last crystals and mother liquor and acetylating the mixture (4.8 mg). The product formed was then crystallized and a constant $^{3}H/^{14}C$ ratio was attained in the third crystals and mother liquor (Table 55).

From Pool VIII (Figure 30, Table 52), a residue was obtained which was applied on a 45 g Celite column using system F. Five-ml fractions were collected and the distribution of radioactive material eluted from the column is shown in Figure 32. Table 56 gives the weight and dpm of the pooled fractions.

Table 56

Weight and Radioactivity of Pooled Fractions Obtained After Celite Column Chromatography of the Residue of Pool VIII (Figure 30, Table 52)

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	70-99	10.6	7.00×10^{3}	6.60×10^{3}	1.1
II	100-130	9.8	6.37×10^{4}	8.80×10^{3}	7.2
Strippings		19.2	1.85×10^{4}	4.60×10^{3}	4.0

The material from Pool II (Figure 32, Table 56) was chromatographed on paper in system Y for 4 hours and then in system X for 27 1/2 hours. In both systems a single peak of radioactive material, which migrated with the same mobility as standard 15α-hydroxyestriol, was obtained. The eluted material from the last chromatogram weighed 1.0 mg and had 4.43×10^{4} dpm of ^{3}H as well as 5.10×10^{3} dpm of ^{14}C. It was

Table 55

Proof of Radiochemical Purity of 15α-Hydroxyestradiol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject BB

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestradiol						
1	2020	97	20.8	1520	220	6.9
2	1990	86	23.1	1920	120	16.0
3	1980	87	22.7	1920	90	21.1
Calculated	1680*	160*				
15α-Hydroxyestradiol triacetate						
1	1390	60	23.2	2230	240	9.3
2	1350	60	22.5	1670	90	18.5
3	1350	60	22.5	1380	60	23.0
Calculated	1380**	60**				

*A total of 25,550 dpm of ^{3}H and 2,300 dpm of ^{14}C was mixed with 9.45 mg of carrier 15α-hydroxyestradiol prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 15α-hydroxyestradiol and adjusting for the change in molecular weight.

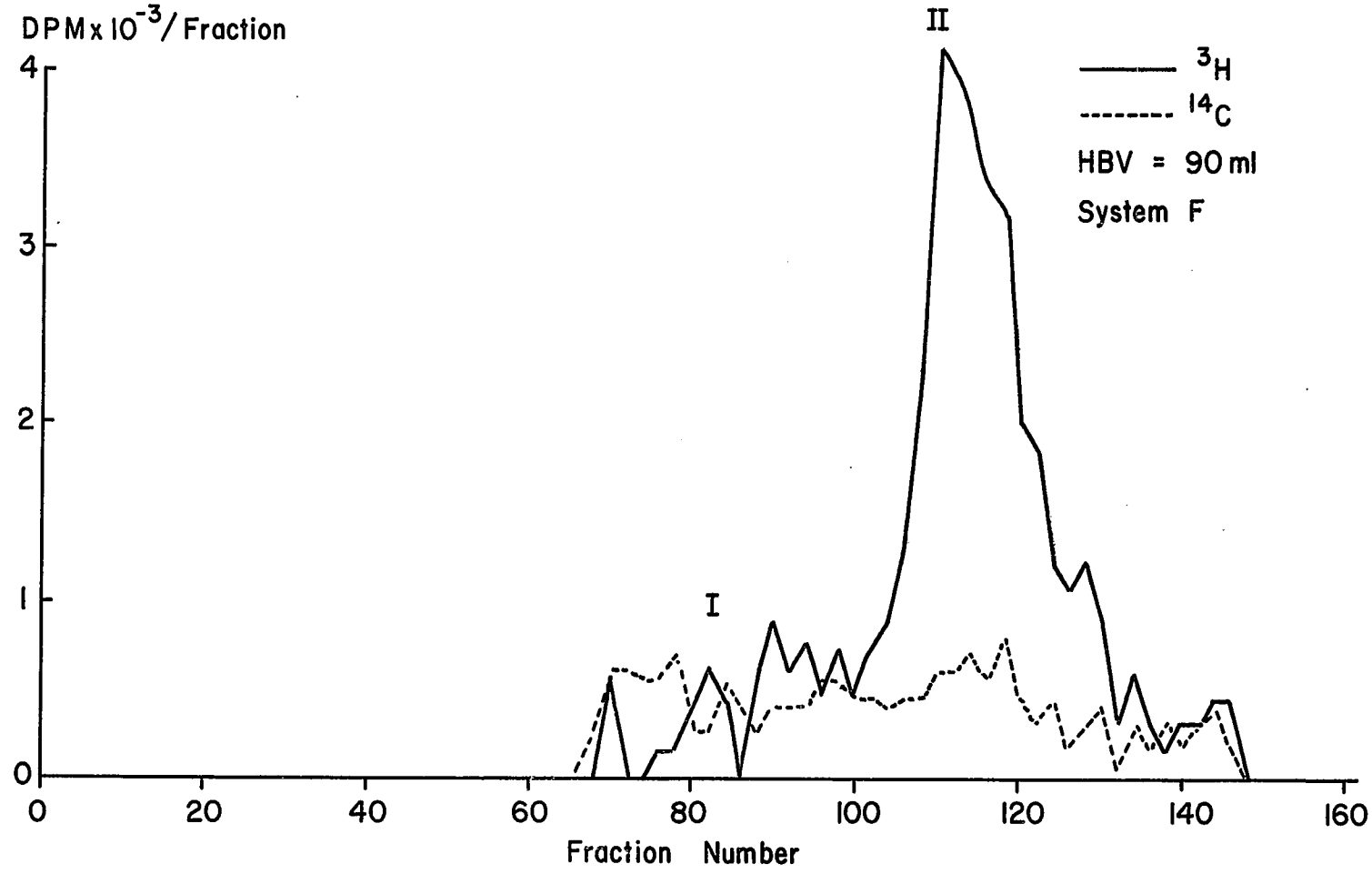


Fig. 32. Celite column chromatography of residue of Pool VIII (Fig. 30, Table 52).

combined with 9.70 mg of carrier 15α-hydroxyestriol and the mixture was crystallized to constant specific activity as shown in Table 57. The derivative formed by acetylation of the third crystals (4.0 mg) was crystallized and the radiochemical homogeneity of the metabolite was established (Table 57).

Table 57

Proof of Radiochemical Purity of 15α-Hydroxyestriol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject BB

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestriol						
1	3550	380	9.3	4890	570	8.4
2	3540	380	9.4	3710	400	9.2
3	3590	380	9.4	3490	370	9.3
Calculated	4580*	490*				
15α-Hydroxyestriol tetraacetate						
1	2490	260	9.4	2250	240	9.2
2	2460	260	9.4	2430	270	9.1
Calculated	2310**	250**				

*A total of 44,400 dpm of ^{3}H and 4,800 dpm of ^{14}C was mixed with 9.70 mg of carrier 15α-hydroxyestriol prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 15α-hydroxyestriol and adjusting for the change in molecular weight.

II. Subject EW

In order to confirm the results just described, a mixture of 1.25×10^7 dpm of ^{3}H-15α-hydroxyandrostenedione (specific activity 1.66×10^6 dpm/μg) and 3.06×10^6 dpm of ^{14}C-15α-hydroxydehydroisoandrosterone (specific activity 4.93×10^4 dpm/μg), $^3H/^{14}C = 4.1$, was administered to subject EW. This subject was in the 28th week of gestation and was undergoing her third IUT. She was in her seventh pregnancy, the sixth being a 24-week stillbirth due to erythroblastosis fetalis. Her previous intrauterine transfusions were at 23 and 24 weeks of gestation. The fetal heart was normal at discharge. About one hour after the injection, the first voiding of approximately 200 ml of urine was inadvertently lost.

Table 58 shows the volume of each day's urine and the dpm of ^{3}H and ^{14}C recovered therein. After pooling the urine, the unconjugated steroids were not extracted in this study. The weight and radioactivity of the fractions representing the sulfates and glucosiduronates are given in Table 59.

The glucosiduronate extract was purified on a 500 g silica gel column and the urinary steroids were eluted with increasing concentrations of ethanol in methylene dichloride. Figure 33 shows a plot of the radioactivity eluted from the column versus the fraction number, and Table 60 gives the weight and dpm of ^{3}H as well as ^{14}C of the material in the various pools of fractions from this column.

The material from Pool III (Figure 33, Table 60) was chromatographed on a 100 g Celite column (HBV = 200 ml) using system D. Ten-ml fractions were collected, and a single peak of radioactive material was eluted in the fourth and fifth hold-back volumes. The residue from this

Table 58

Radioactivity Excreted in the Urine Following the Administration of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone in Subject EW

Day	Volume (ml)	^{3}H (dpm)	% of I.D.*	^{14}C (dpm)	% of I.D.	$^{3}H/^{14}C$
1	1840	2.47×10^{6}	19.8	3.88×10^{5}	12.7	6.4
2	1880	6.28×10^{5}	5.0	1.18×10^{5}	3.8	5.3
3	1520	2.26×10^{5}	1.8	2.05×10^{4}	0.7	11.0
4	880	2.16×10^{5}	0.7	2.02×10^{4}	0.7	10.7
5	780	8.77×10^{4}	29.0	7.80×10^{3}	0.2	11.2
TOTAL	6900	3.63×10^{6}	29.0	5.54×10^{5}	18.1	6.5

*I.D. = Injected Dose

Table 59

Weight and Radioactivity of the Conjugated Fractions From the Pregnancy Urine of Subject EW

Fraction	Weight (g)	^{3}H (dpm)	% of dpm in Urine	^{14}C (dpm)	% of dpm in Urine	$^{3}H/^{14}C$
Sulfate	4.2	1.34×10^{5}	3.7	7.60×10^{3}	1.4	17.7
Glucosi-duronate	2.2	1.28×10^{6}	35.3	2.61×10^{5}	47.1	4.9
TOTAL		1.41×10^{6}	39.0	2.68×10^{5}	48.5	5.3

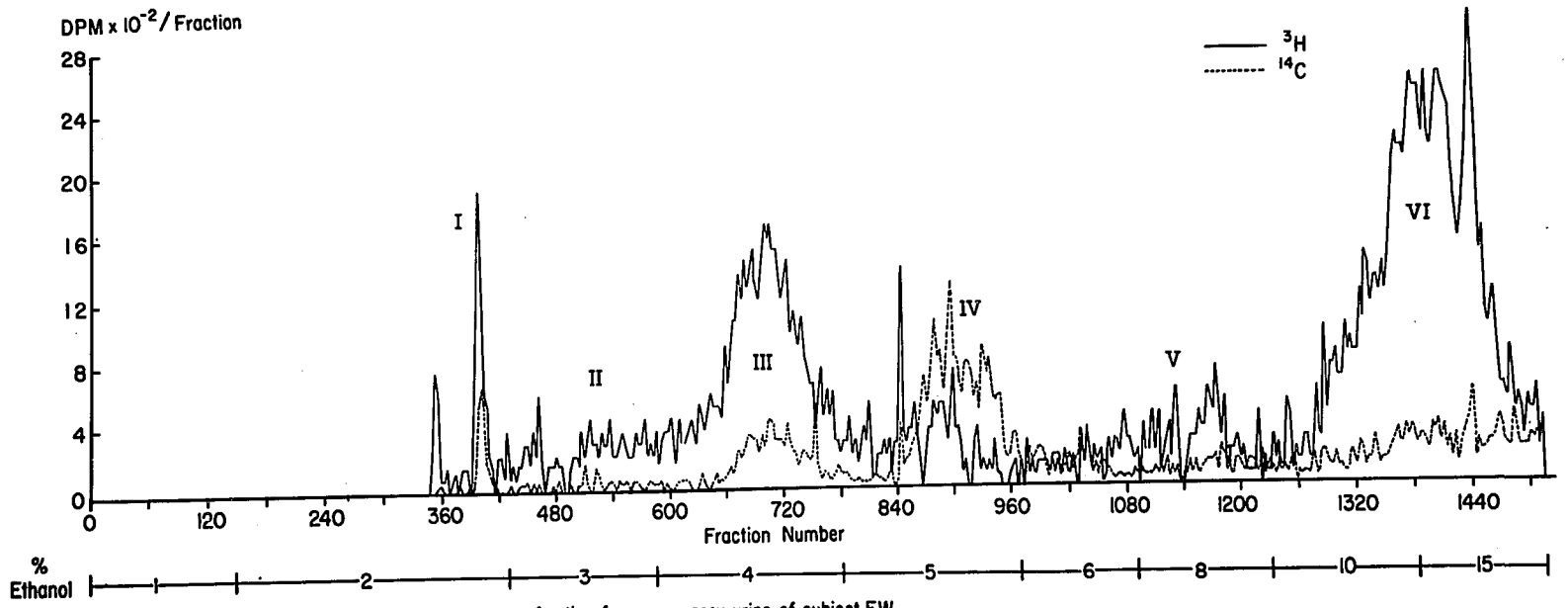


Fig. 33. Silica gel column chromatography of glucosiduronate fraction from pregnancy urine of subject EW.

Table 60

Weight and Radioactivity of the Pooled Fractions Obtained After Silica Gel Column Chromatography of the Glucosiduronate Extract From the Pregnancy Urine of Subject EW

Pool	Fraction No.	Weight (mg)	^{3}H (dpm)	^{14}C (dpm)	$^{3}H/^{14}C$
I	394-414	302.8	3.30×10^{4}	7.65×10^{3}	4.3
II	415-651	417.8	5.19×10^{4}	9.40×10^{3}	5.5
III	652-846	179.9	1.28×10^{5}	3.05×10^{4}	4.2
IV	847-1059	158.4	6.33×10^{4}	9.31×10^{4}	0.7
V	1060-1275	115.7	4.24×10^{4}	1.89×10^{4}	2.2
VI	1276-1512	103.4	2.83×10^{5}	1.34×10^{5}	2.1
Strippings		143.7	8.05×10^{4}	2.62×10^{4}	3.1

peak weighed 7.4 mg and contained 4.74×10^{4} dpm of ^{3}H and 1.64×10^{4} dpm of ^{14}C. It was further purified by paper chromatography, first in system O for 22 1/2 hours and then in system M for 4 hours. In each system a single radioactive peak migrated with the same mobility as standard 15α-hydroxyandrostenedione and, from the last chromatogram, a residue, weighing 1.9 mg and containing 3.90×10^{4} dpm of ^{3}H and 1.37×10^{4} dpm of ^{14}C, was obtained. This material was mixed with 25.25 mg of carrier 15α-hydroxy-androstenedione and the mixture was crystallized to constant specific activity as shown in Table 61. A derivative was formed by acetylation, reduction with $NaBH_4$ and oxidation with DDQ of 14.3 mg of the final crystals. The crude product was chromatographed on a 4 g alumina column and elution with 0.5% ethanol in benzene gave a residue which weighed 11.1 mg and contained 1.27×10^{4} dpm of ^{3}H and 3.35×10^{4} dpm of ^{14}C. It

Table 61

Proof of Radiochemical Purity of 15α-Hydroxyandrostenedione Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject EW

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyandrostenedione						
1	1470	530	2.8	1260	450	2.8
2	1490	540	2.7	1420	500	2.8
3	1480	540	2.8	1450	510	2.8
Calculated	1550*	540*				
15α-Acetoxytestosterone						
1	1350	470	2.9	900	310	2.9
2	1380	490	2.8	1340	450	2.9
Calculated	1290**	470**				

*A total of 39,050 dpm of ^{3}H and 13,700 dpm of ^{14}C was mixed with 25.25 mg of carrier 15α-hydroxyandrostenedione prior to crystallization.

**This value was computed by using the final specific activity of the crystals of 15α-hydroxyandrostenedione and adjusting for the change in molecular weight.

was then crystallized and the radiochemical purity of the isolated 15α-hydroxyandrostenedione was established (Table 61). The infrared spectrum (KBr) of this material was identical to that of 15α-acetoxytestosterone.

The material in Pool IV (Figure 33, Table 60) was purified on a 75 g Celite column (HBV = 150 ml) using system D. Ten-ml fractions were collected and a single peak of radioactive material was eluted in the sixth to eighth hold-back volumes. The pooled fractions representing this peak gave a residue which had a weight of 8.6 mg and contained 2.60×10^3 dpm of ^3H and 4.37×10^4 dpm of ^{14}C. This material was chromatographed on paper in system O for 40 hours and, on scanning, a single radioactive zone, which migrated approximately 5 cm ahead of standard 15α-hydroxydehydroisoandrosterone, was detected. This material was not identified because it could not be matched to a known standard.

The residue of Pool VI (Figure 33, Table 60) was also purified by Celite column chromatography. It was applied on a 75 g column (HBV = 150 ml) in system F and 10 ml fractions were collected. A broad radioactive peak was eluted from the column and the residue obtained had a weight of 17.6 mg and contained 2.52×10^5 dpm of ^3H as well as 3.37×10^4 dpm of ^{14}C. Chromatography of this material on paper in system X for 23 hours yielded two radioactive materials. The less polar material migrated with the same mobility as standard 15α-hydroxyestradiol, whereas the more polar material had the same mobility as 15α-hydroxyestriol. The less polar residue was further chromatographed on paper in system M for 40 hours and it resolved into two radioactive bands. Only the zone which migrated with the mobility of 15α-hydroxyestradiol was processed further, and the residue obtained after elution weighed 0.5 mg and contained

2.50×10^4 dpm of ^3H and 1.85×10^3 dpm of ^{14}C. The polar material obtained in the first chromatogram was purified on paper using system W for 4 hours and it again migrated as a single radioactive peak corresponding in mobility to 15α-hydroxyestriol. On elution, a residue weighing 1.50 mg and containing 1.53×10^5 dpm of ^3H and 1.08×10^4 dpm of ^{14}C was obtained. Each of the residues was then mixed with the appropriate carrier steroid and crystallized to constant specific activity as shown in Tables 62 and 63. Further evidence for the radiochemical homogeneity of 15α-hydroxyestradiol and 15α-hydroxyestriol was obtained after the preparation and subsequent crystallization of the acetates (Tables 62 and 63).

Table 62

Proof of Radiochemical Purity of 15α-Hydroxyestradiol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject EW

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestradiol						
1	2590	130	19.9	2090	240	8.7
2	2560	130	19.7	2330	160	14.6
3	2570	130	19.8	2590	140	18.5
Calculated	2580*	180*				
15α-Hydroxyestradiol triacetate						
1	2350	130	18.1	1160	200	5.8
2	1940	90	21.5	1890	100	18.9
3	1920	90	21.3	2000	90	22.2
Calculated	1790**	90**				

*A total of 25,400 dpm of ^{3}H and 1,800 dpm of ^{14}C was mixed with 9.85 mg carrier 15α-hydroxyestradiol prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 15α-hydroxyestradiol and adjusting for the change in molecular weight.

Table 63

Proof of Radiochemical Purity of 15α-Hydroxyestriol Isolated From the Glucosiduronate Fraction of the Pregnancy Urine of Subject EW

	Specific Activity (dpm/mg)					
	Crystals			Mother Liquor		
Crystallization	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
15α-Hydroxyestriol						
1	12,130	870	14.0	12,040	800	15.0
2	12,050	850	14.1	11,450	780	14.6
3	12,300	890	13.8	12,040	860	14.0
Calculated	14,700*	980*				
15α-Hydroxyestriol tetraacetate						
1	8,580	520	16.6	6,010	340	17.8
2	8,580	530	16.3	8,290	510	16.2
Calculated	7,920**	570**				

*A total of 163,950 dpm of ^{3}H and 10,950 dpm of ^{14}C was mixed with 11.15 mg of carrier 15α-hydroxyestriol prior to crystallization.

**This value was calculated by using the final specific activity of the crystals of 15α-hydroxyestriol and adjusting for the change in molecular weight.

DISCUSSION

Because pregnenolone is a key intermediate in the formation of steroids in the human fetus and a knowledge of its metabolism was available, we chose to study its disposition in the feto-placental unit of the Rhesus monkey in order to determine whether a parallel pattern in metabolism exists in this species. The experimental design used to carry out this study entailed the injection of ^{14}C-pregnenolone into the umbilical vein with the fetus in utero, a procedure previously employed in studying the metabolism of this steroid (72). The umbilical circulation was left intact for 3 1/2 minutes and, at the end of this period, a number of fetal tissues as well as the placenta were taken for analysis. It is evident from the data presented in Table 7 that most of the radioactivity in these tissues was in an ether-soluble form. Solomon et al (72) injected ^{14}C-pregnenolone into the umbilical vein in a subject in the thirteenth week of gestation and found that a sizable portion of the radioactivity in the adrenals, liver, lung and intestine was water soluble, whereas most of the radioactivity in the kidney, placenta and the residue was in an ether-soluble form. Metabolites of pregnenolone isolated from the tissues in the present study are shown in Table 64. The numbers in this table represent the percentage of the total radioactivity in each tissue found in the individual metabolite after its radiochemical homogeneity had been established. Since the amounts of radioactivity measured during the purification of the metabolites were not corrected for losses, the percentages shown represent minimal values.

Table 64

Metabolites Isolated From the Tissues of the Rhesus Monkey Fetus and Placenta Following the Injection of ^{14}C-Pregnenolone

	Percentage of Total Radioactivity in Each Tissue		
Tissue	Pregnenolone	3β-Hydroxy-5α-pregnan-20-one	Progesterone
Adrenal	57.20	-	-
Liver	9.30	0.24	0.08
Placenta	15.50	0.20	-
Heart	79.91	0.09	-
Intestine	73.80	0.22	-
Brain	57.00	-	-
Cord	67.10	-	-
Kidney	60.84	-	-
Lung	65.60	-	-

Pregnenolone was isolated from all the tissues examined. The low percentage recovery of pregnenolone in the liver and placenta indicates that these tissues are very active sites of the metabolism of pregnenolone in comparison to the other tissues analyzed. Leung and Solomon (23) found that the fetal liver and placenta of the Rhesus are very active sites of the metabolism of progesterone, and Solomon et al (72) isolated large amounts of unmetabolized pregnenolone from the intestine, kidney, liver, lung, placenta and residue of the human fetus.

In these studies, minute amounts of 3β-hydroxy-5α-pregnan-20-one were isolated from the heart, intestine, liver and placenta. In the study by Solomon and Leung (23) this compound was the major metabolite of

progesterone, isolated from the Rhesus monkey placenta. From this result, Leung (73) suggested that the conclusions drawn by Short and Eckstein (11) regarding the low level of progesterone in the placenta of the Rhesus monkey may not be valid. In the study of the human fetus (72), 3β-hydroxy-5α-pregnan-20-one was not found in the fetal tissues or the placenta, following the administration of pregnenolone.

A minute amount of progesterone was also isolated from the fetal liver. The isolation of this metabolite from no other tissue but the liver indicates the presence of the 3β-hydroxysteroid dehydrogenase enzyme system in the fetal liver of the Rhesus monkey. It is interesting to note that in the study of the metabolism of pregnenolone in the human fetus (72), the only tissue from which metabolites have a Δ^4-3-ketone function were isolated was the placenta. Human fetal tissues at mid-pregnancy seem to be completely devoid of the 3β-hydroxysteroid dehydrogenase enzyme system for the metabolism of pregnenolone.

A considerable number of metabolites in Experiment 1 could not be identified; this was especially true in the fetal liver. In several instances these compounds had the same chromatographic mobility on paper as known steroids but were found to be radiochemically impure. In the human fetus (72), the following metabolites of pregnenolone were identified: 20α-dihydropregnenolone from the liver and the residue; 16α-hydroxypregnenolone from the liver; dehydroisoandrosterone from the adrenal. Progesterone as well as 20α-dihydroprogesterone were isolated from the placenta. In addition, a number of conjugated metabolites were isolated from the human fetal tissues and these are as follows: pregnenolone sulfate from the adrenal, intestine, liver, lung, kidney and residue;

20α-dihydropregnenolone-3-sulfate from the liver; 17α-hydroxypregnenolone-3-sulfate from the adrenals; dehydroisoandrosterone sulfate from the adrenals as well as the residual fetal tissues. In the present study the unknown metabolites from the water soluble portion of the liver migrated as conjugated steroids in paper chromatographic systems but none of these metabolites could be identified. Thus, it is evident that conjugation of pregnenolone or 3β-hydroxy-5α-pregnan-20-one with sulfuric acid does not occur to any extent in the fetal tissues and placenta of the Rhesus monkey.

It is obvious that to draw any conclusion from the data of one experiment is not very satisfactory. However, it seems permissible to state that, with the exception of the liver, the midterm fetal tissues of the Rhesus monkey do not metabolize pregnenolone to any significant extent. Furthermore, in view of the large number of unidentifiable compounds in the fetal liver, it appears that in the pregnant Rhesus, pregnenolone may be metabolized to a number of products by pathways which may be different from those in the human fetus. The unknown metabolites may be identified if an educated guess can be made concerning their structure and if they could be prepared in sufficient quantities to establish their identity. Finally, on the basis of the results obtained in this study, it appears that the pregnant Rhesus monkey would serve as a poor model for the human feto-placental unit. However, further studies must be carried out to confirm these results and to obtain more knowledge regarding the metabolism of a large class of steroids in the Rhesus monkey.

In Experiment 2, the synthesis of 15α-hydroxydehydroisoandrosterone was undertaken as the first step in the study of the role of this compound in the formation of 15α-hydroxyestrogens during pregnancy. Since

the formation of estriol in pregnancy involves C-19 steroids possessing the Δ^5-3β-hydroxy group or the corresponding sulfate, it seemed quite likely that some C-19 15α-hydroxy-Δ^5-3β-hydroxy steroids may be involved in the formation of 15α-hydroxylated estrogens. However, no studies had been done with a steroid such as 15α-hydroxydehydroisoandrosterone, primarily because it was not available in a labeled or non-labeled form. Consequently, the synthesis of this compound was undertaken.

The most common method for the introduction of a 15α-hydroxy group into a steroid nucleus is by microbiological oxidation (74). This method was unsuccessful in the present study due to the fact that most microorganisms known to 15α-hydroxylate steroids also possess the 3β-hydroxysteroid dehydrogenase Δ^5-isomerase enzyme system. Thus, when dehydroisoandrosterone was incubated with these microorganisms, the product isolated was 15α-hydroxyandrostenedione and not 15α-hydroxydehydroisoandrosterone (75). Since 15α-hydroxyandrostenedione can be prepared very easily by microbiological hydroxylation, a method which could convert 15α-hydroxyandrostenedione to 15α-hydroxydehydroisoandrosterone was developed. Figure 34 shows the structures of the various intermediates used in the synthesis of 15α-hydroxydehydroisoandrosterone and its derivatives.

The first step in the synthesis of 15α-hydroxydehydroisoandrosterone (V) was the treatment of 15α-hydroxyandrostenedione (I) with acetic anhydride and acetyl chloride by a procedure described by Fukushima and Teller (76); this reaction yielded 3,15α-diacetoxyandrost-3,5-dien-17-one (II). The yield of this enol acetate was 66% and its structure was deduced from its infrared and mass spectra described earlier.

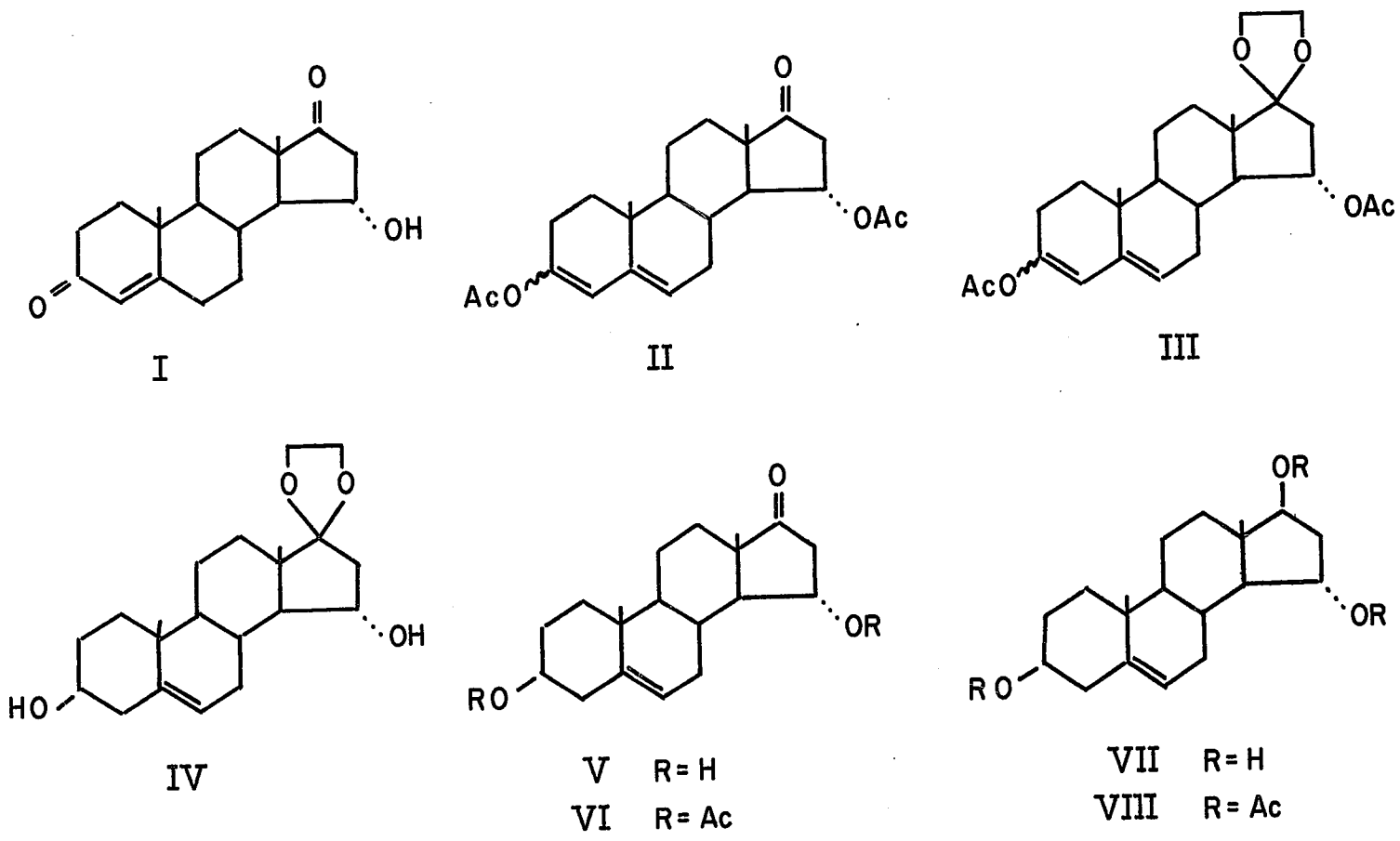


Fig. 34. Structures of various steroid intermediates in the synthesis of 15α-hydroxydehydroisoandrosterone and its derivatives.

Initially, several attempts to prepare 15α-hydroxydehydroisoandrosterone from the enol acetate (II) were unsuccessful (77). Reduction of the latter compound with $NaBH_4$ in isopropyl alcohol and water as described by Fukushima and Dobriner (78) gave a product which had the 3β-hydroxy-Δ^5 configuration, but the ketone group at the C-17 position of the product was reduced; the product was shown to be 15α-acetoxyandrost-5-ene-3β,17β-diol. The same compound was obtained by using anhydrous isopropanol. Treatment of the enol acetate (II) in a solution of $NaBH_4$ in pyridine as described by Kupfer (79) yielded only ultraviolet absorbing products. Formation of a complex with digitonin and 15α-acetoxyandrost-5-ene-3β,17β-diol, followed by oxidation of the digitonide with a solution of chromic acid in acetic acid and subsequent cleavage of the digitonide by a method originally described by Bergmann (80), yielded two Oertel positive products which could not be identified. Finally, an attempt to prepare 15α-hydroxydehydroisoandrosterone by oxidation of the C-17 hydroxyl group of androst-5-ene-3β,15α,17β-triol (VII), using a placental 17β-hydroxysteroid dehydrogenase, was unsuccessful. The substrate (VII) was readily obtained by hydrolysis of 15α-acetoxyandrost-5-ene-3β,17β-diol with 15% HCl as described by Ladany and Finkelstein (81).

Adequate amounts of 15α-hydroxydehydroisoandrosterone were synthesized by protecting the 17-ketone group of the enol acetate (II) through the formation of the ketal, and reducing the product formed with $NaBH_4$ and$LiAlH_4$ prior to cleavage of the ethylene ketal at carbon 17. Initially, the 17-ketone group was ketalized by using the procedure (82) whereby the steroid is refluxed with a mixture of benzene, ethylene glycol and p-toluenesulfonic acid in an apparatus fitted with a water separator.

Although this method gave approximately a 5% yield of 15α-hydroxydehydroisoandrosterone, many side products were obtained and the purification procedure was very time consuming. Preparation of the ketal by a modification of the procedure originally described by Antonucci et al (83,84) was found to give very few side products at the end of the sequence of reactions. This method involved dissolving the enol acetate (II) in benzene and then adding ethylene glycol and p-toluenesulfonic acid, followed by very slow vacuum distillation (0.1 micron) at a temperature of 38°C for a period of 4 1/2 hours. Heating the mixture at higher temperatures resulted in decomposition of the enol acetate (II), and carrying out the reaction for a longer period of time did not increase the yield of 15α-hydroxydehydroisoandrosterone. Attempts to purify the crude ketal failed. However, the infrared spectrum (CS_2) of the crude material clearly showed the characteristic bands of the enol acetate at 1740, 1670, 1640, 1240 and 1215 cm^{-1} as well as the C-O band of the ethylene ketal at 945 cm^{-1}. The positions of these bands were compatible with the structure of 3,15α-diacetoxyandrost-3,5-dien-17-one cyclic ethylene ketal (III).

The crude ketal (III) was then reduced with $NaBH_4$ giving rise to the 3β-hydroxy-Δ^5 group. During this reaction it was observed that partial hydrolysis of the 15α-acetate group had occurred, therefore the product was thereafter treated with $LiAlH_4$ to completely remove the 15α-acetate group. Direct reduction of the ketal (III) with $LiAlH_4$ gave poorer yields of 15α-hydroxydehydroisoandrosterone at the end of the sequence of reactions. The resulting crude 3β,15α-dihydroxyandrost-5-en-17-one cyclic ethylene ketal (IV) was not purified and was used as such in the next reaction.

The last step in the synthesis of 15α-hydroxydehydroisoandrosterone was the cleavage of the ethylene ketal at carbon 17. This was done by refluxing a solution of the ketal (IV) in aqueous acetone and p-toluenesulfonic acid, and the product obtained was purified by chromatography and crystallization. After two crystallizations, the overall yield of 15α-hydroxydehydroisoandrosterone from 15α-hydroxyandrostenedione was 6%.

The structure of 15α-hydroxydehydroisoandrosterone was verified by infrared, mass and elemental analyses and by converting it enzymatically to 15α-hydroxyandrostenedione. NMR analysis of 15α-hydroxydehydroisoandrosterone was carried out, but its spectrum was not reported in the previous section of this thesis since the analysis was done using pyridine as the solvent, instead of deuteriochloroform and, consequently, the NMR spectrum was very difficult to interpret. The change of solvent was necessitated because of the insolubility of 15α-hydroxydehydroisoandrosterone in deuteriochloroform.

Further verification of the structure of 15α-hydroxydehydroisoandrosterone was obtained by the study of its derivatives, namely, 15α-hydroxydehydroisoandrosterone diacetate (VI) androst-5-ene-3β,15α,17β-triol (VII) and androst-5-ene-3β,15α,17β-triol triacetate (VII). All the compounds gave infrared spectra that were compatible with their structures. The NMR spectrum of 3β,15α-diacetoxyandrost-5-en-17-one indicated that the chemical shifts for the C_{18} and C_{19} angular methyls were in full agreement with the calculated values, and the signals for the two acetate methyls (CH_3COO) and the protons at C-3, C-6 and C-15 were present at positions compatible with the structure of this compound. The mass spectra of the diacetate (VI) and triacetate (VII) showed evidence of thermal

decomposition in the ion source (290°C), as an apparent molecular ion was observed at m/e 328 and 372, respectively. Since pyrolytic elimination of acetic acid is a common process in the case of steroidal acetates, if the double bond formed is conjugated with the double bond in the steroid ring, the mass spectra are still in agreement with the respective proposed structures of these compounds.

High specific activity 4-^{14}C-15α-hydroxydehydroisoandrosterone was prepared from 4-^{14}C-15α-hydroxyandrostenedione by using the method described for the synthesis of unlabeled 15α-hydroxydehydroisoandrosterone. The reactions were essentially performed in the same manner except that the time of ketalization was reduced to two hours. The overall yield of ^{14}C-15α-hydroxydehydroisoandrosterone after five chromatographic purification steps was 8% and the radiochemical purity of this compound, as determined by isotope dilution, was found to be over 97%.

In the third experiment, very small amounts of 15α-hydroxydehydroisoandrosterone were isolated from late pregnancy urine. The difficulty in isolating a steroid present in the urine in minute amounts is mainly due to the huge volumes of urine which have to be processed in order to obtain sufficient quantities of the compound for chemical identification. This obstacle was in part circumvented in these studies by the use of the isotope dilution procedure (65). In this method small amounts of ^{14}C-15α-hydroxydehydroisoandrosterone of known specific activity were added to the urine prior to the hydrolysis of the urinary steroid conjugates and extraction of the resulting unconjugated steroids. The neutral fraction obtained from the extraction was chromatographed first on a large silica gel column, then on a Celite column and finally on paper.

The purified extract containing the ^{14}C-15α-hydroxydehydroisoandrosterone was acetylated with ^{3}H-acetic anhydride of known specific activity and, after mixing the acetylated product with the appropriate carrier steroid, the mixture was crystallized to constant specific activity. The crystallization procedure was repeated after the formation of a suitable derivative. From the final $^{3}H/^{14}C$ ratio of the derivative and the amount of radioactivity originally added to the urine, it was possible to calculate the amount of 15α-hydroxydehydroisoandrosterone excreted in the urine. In the first isolation study (15 day urine pool), it was calculated that 3.20 μg/day of this compound was excreted in the urine during late pregnancy.

In the other isolation study (5 day urine pool) a slightly different approach was taken before acetylating the purified extract containing ^{14}C-15α-hydroxydehydroisoandrosterone with ^{3}H-acetic anhydride. The extract was first reduced with $NaBH_4$ and the product was purified by paper chromatography and then acetylated with tritiated acetic anhydride. The specific activity of ^{14}C-15α-hydroxydehydroisoandrosterone was also determined in this manner. This approach was used because it soon became apparent that whenever ^{14}C-15α-hydroxydehydroisoandrosterone of high specific activity was acetylated with either labeled or unlabeled acetic anhydride and the radiochemical purity of the acetylated product was determined by isotope dilution, the specific activity with respect to ^{14}C was unusually high in the first (and sometimes second) mother liquor (Tables 27 and 28). In the course of attempting to solve this problem, the ^{14}C-labeled impurity was found to range from 8% to 20%, even though the same stock solution of ^{14}C-15α-hydroxydehydroisoandrosterone was used.

This impurity was not observed when low specific activity ^{14}C-15α-hydroxydehydroisoandrosterone was acetylated as seen in Table 26. The reason for this discrepancy is not fully understood. As a result, in the second isolation study the isolated material containing ^{14}C-15α-hydroxydehydroisoandrosterone was first converted to ^{14}C-androst-5-ene-3β,15α,17β-triol prior to acetylation with ^{3}H-acetic anhydride. The acetylated product was then combined with carrier androst-5-ene-3β,15α,17β-triol triacetate and the mixture was crystallized to constant specific activity as shown in Table 29. No derivative of this compound was prepared, since an insufficient amount of crystals and mother liquor remained for this purpose. Consequently, from the ^{3}H/^{14}C ratio of the final crystals of the androst-5-en-3β,15α,17β-triol triacetate (Table 29) and the amount of 15α-hydroxydehydroisoandrosterone originally added to the urine, the urinary excretion rate of this steroid was computed to be 1.76 µg/day.

The addition of small amounts of ^{14}C-15α-hydroxydehydroisoandrosterone of high specific activity in the isolation studies greatly facilitated the detection of minute amounts of the unlabeled material, since the radioactivity was easily followed in every chromatographic step by the counting of fractions from columns and by the scanning of paper chromatograms. Moreover, since the labeled steroid added to the urine was mixed with the excreted urinary steroid, losses occurring during purification could be corrected and quantitative results were obtained. However, possible losses due to the degree and specificity of the hydrolytic procedure were not taken into account by this method. In both isolation studies, Glusulase (a mixture of sulfatase and β-glucuronidase) was used for the hydrolysis of the urinary steroid conjugates. However,

other conjugated forms of urinary 15α-hydroxydehydroisoandrosterone cannot be excluded. Optimal quantification can only be achieved if the labeled steroid which is added to the urine is in the form of the conjugate(s) excreted.

The addition to the urine of a known amount of ^{14}C-15α-hydroxydehydroisoandrosterone was a critical step in the quantitative procedure just described, since an error in this figure would lead to inaccuracies in the final calculation. Consequently, when an aliquot of the radioactive material was added to the urine, at the same time three aliquots of this material were taken for counting. In each case, the aliquots counted did not differ by more than 2%. Thus, the error of pipetting the radioactive material to the urine was less than 2%.

15α-Hydroxydehydroisoandrosterone is the first C-19 15α-hydroxylated steroid with a 3β-hydroxy-Δ^5 structure identified in man. Since recent studies have shown that urinary 15α-hydroxysteroids excreted during pregnancy are almost exclusively of fetal origin, it is quite likely that the excretion of 15α-hydroxydehydroisoandrosterone is also very closely correlated with the function of the feto-placental unit. Furthermore, it is tempting to speculate that this steroid is a fetal metabolite of dehydroisoandrosterone or its sulfate. This speculation is supported not only by the finding that fetal tissues are able to carry out 15α-hydroxylations of steroids, but also by the evidence that the de novo synthesis of dehydroisoandrosterone sulfate in the fetal adrenal is rather high (72).

The presence of 15α-hydroxydehydroisoandrosterone in late pregnancy urine raised the possibility that this compound may be an important procursor of 15α-hydroxylated estrogens found in the urine

of pregnant women during the late stages of gestation. As mentioned earlier, recent studies by YoungLai and Solomon (28,46) have demonstrated that 15α-hydroxyandrostenedione administered to women in late pregnancy is a good precursor of 15α-hydroxyestradiol and 15α-hydroxyestriol. Since the placenta contains an active 3β-hydroxysteroid dehydrogenase-Δ^5-isomerase enzyme system, it seemed likely that in pregnancy 15α-hydroxydehydroisoandrosterone might be rapidly converted to 15α-hydroxyandrostenedione and then aromatized to form 15α-hydroxylated estrogens. Consequently, in Experiment 4, tracer doses of ^{3}H-15α-hydroxyandrostenedione and ^{14}C-15α-hydroxydehydroisoandrosterone were administered intravenously to two subjects in the third trimester of pregnancy. Since 15α-hydroxylation occurs in the fetus, this approach can be used to study the formation of 15α-hydroxyestrogens, but the difficulty in such studies is that the rate of entry of the injected precursors into the feto-placental unit may be different. Although this somewhat complicates the results, nevertheless, valuable information can still be obtained from such studies.

It is interesting to note that the excretion of tritium in the 4 day urine collection of both subjects was approximately 70% of the injected dose (Tables 31 and 40), whereas a somewhat lower percentage (55% and 45%) of the ^{14}C dose was recovered from these urines. In both subjects, over 90% of the excreted tritium and carbon-14 were present in the urine of the first day, indicating that the urinary metabolites resulting from the injected steroids were cleared rapidly.

The urinary conjugates were cleaved first by solvolysis and then by β-glucuronidase hydrolysis. It is therefore possible that the glucosiduronate fraction contained some metabolites which were excreted

in the urine conjugated with both sulfuric and glucosiduronic acids. In both subjects most of the radioactivity was found in the glucosiduronate fraction and a small percentage of the injected dose was found in the sulfate fraction (Tables 32 and 41).

Four metabolites were isolated from the urine of each subject in Experiment 4. Since only small amounts of these metabolites were isolated, their identification was achieved by the isotopic dilution procedure. Table 65 shows the metabolites isolated from the urine of subjects TL and MP, the final $^{3}H/^{14}C$ ratios of these metabolites, and the radioactivity in each metabolite, expressed as a percentage of the injected dose, after its radiochemical purity was established. It should be noted that losses occurring during the isolation and purification of these metabolites were not taken into consideration in determining the percentages in Table 65, hence the numbers given are minimal values. All the metabolites were isolated from the glucosiduronate fraction of the urine. As mentioned earlier, this fraction could also contain metabolites conjugated with both sulfuric and glucosiduronic acids. Attempts to identify some of the radioactive material, obtained after silica gel column chromatography of the sulfate fraction from the urine of subject TL (Figure 23, Table 33), were unsuccessful.

As can be seen from the data in Table 65, the injected substrates were recovered from the urine of both subjects. The urinary 15α-hydroxy-androstenedione contained both labels, indicating that 15α-hydroxydehydro-isoandrosterone was converted to this steroid.

Only small amounts of the injected 15α-hydroxydehydroisoandrost-erone were recovered from the urines of both subjects. This result could

Table 65

Urinary Metabolites Isolated in Experiment 4 Following the Injection of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone

Metabolite	Subject TL ($^{3}H/^{14}C$ = 5.2)			Subject MP ($^{3}H/^{14}C$ = 5.1)		
		% of Injected Dose			% of Injected Dose	
	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C
15α-Hydroxyandrostenedione	10.7	12.60	6.21	16.5	16.61	5.03
15α-Hydroxydehydroisoandrosterone	0.0	0.00	1.42	0.0	0.00	3.08
15α-Hydroxyestradiol	15.7	0.15	0.05	23.9	0.05	0.01
15α-Hydroxyestriol	10.3	0.10	0.05	16.6	0.23	0.07
Unknown	0.0*	0.00*	13.06*	0.0*	0.00*	14.28*

*These values were computed from the amount of radioactivity obtained after elution of the unknown metabolite from the last chromatogram.

be explained by the large percentage conversion (13.06% and 14.28%) of this substrate to an unknown metabolite containing only the ^{14}C label. This compound did not separate from 15α-hydroxydehydroisoandrosterone on the silica gel and Celite columns which were used in the purification procedure. However, it was less polar than 15α-hydroxydehydroisoandrosterone in certain paper chromatographic systems and was easily separated from the latter compound. Unfortunately, this metabolite contained insufficient weight for identification.

It is tempting to speculate that the presence of a 15α-hydroxyl group partially interferes with the Δ^5-isomerase enzyme, which would normally play a role in the conversion of a β,γ-unsaturated alcohol to an α,β-unsaturated ketone, and that a metabolite such as 3α,15α-dihydroxyandrost-5-en-17-one is formed. This situation would then be analogous to that found by YoungLai and Solomon (85) following the injection of labeled 16α-hydroxydehydroisoandrosterone into a subject in late pregnancy. These authors found that the major urinary metabolite excreted was 3α,16α-dihydroxyandrost-5-en-17-one. That a 3-keto-5-ene steroid may be an intermediate in the formation of a 3α-hydroxy-5-ene steroid was suggested by the studies of Fukushima et al (86) who demonstrated the conversion of androst-5-ene-3,17-dione to urinary 3α-hydroxyandrost-5-en-17-one. Further studies will be carried out in order to attempt to identify the unknown metabolite.

Table 65 also shows that both substrates were converted to 15α-hydroxyestradiol and 15α-hydroxyestriol, and that 15α-hydroxyandrostenedione was the better precursor for these 15α-hydroxyestrogens. Although the $^{3}H/^{14}C$ ratios of 15α-hydroxyestradiol (15.7) and 15α-hydroxyestriol (10.3),

isolated from the urine of subject TL, would seem to indicate that more 15α-hydroxydehydroisoandrosterone was converted to 15α-hydroxyestriol than to 15α-hydroxyestradiol, the percentage conversion of this substrate to both 15α-hydroxylated estrogens was the same (0.05%). Hence, equal amounts of 15α-hydroxydehydroisoandrosterone were converted to 15α-hydroxyestradiol and 15α-hydroxyestriol in this subject. In the second study (subject MP), however, the $^{3}H/^{14}C$ ratios of the isolated 15α-hydroxyestradiol (23.9) and 15α-hydroxyestriol (16.6), as well as the larger percentage conversion (0.07% vs 0.01%) of 15α-hydroxydehydroisoandrosterone to 15α-hydroxyestriol than to 15α-hydroxyestradiol, indicate that more of the former than the latter compound is formed from 15α-hydroxydehydroisoandrosterone.

Also, as can be seen in Table 65, the percentage conversion of 15α-hydroxyandrostenedione to urinary 15α-hydroxyestradiol in subjects TL and MP was 0.15 and 0.05, whereas the conversion of the same substrate to urinary 15α-hydroxyestriol was 0.10% and 0.23%. Slightly higher conversions of 15α-hydroxyandrostenedione to 15α-hydroxyestradiol (0.29% and 0.48%) and to 15α-hydroxyestriol (0.47% and 0.48%) were obtained in similar studies by YoungLai and Solomon (28).

It was pointed out earlier that an inherent objection to the type of approach used in Experiment 4 is the lack of knowledge regarding the relative amounts of labeled substrates entering the feto-placental unit from the maternal circulation. In order to overrule this criticism, the same substrates were injected into the fetus during an intrauterine transfusion for erythroblastosis fetalis. The data obtained in two such studies in Experiment 5 have only served to strengthen the conclusions drawn from the results of Experiment 4.

In Experiment 5, the processing of the urines, the purification of the extracts and the identification of the isolated metabolites were performed in the same manner as in Experiment 4. Less than 40% of the injected ^{3}H and less than 30% of the administered ^{14}C were recovered from the urines as shown in Tables 49 and 58. Approximately 70% of the total ^{3}H and ^{14}C recovered was found in the first day's urine in both studies. The lower recovery of ^{3}H and ^{14}C in the case of subject EW may be due in part to the fact that the first voiding was lost. The fate of the remainder of the radioactivity is not known, since the urine collection from the fifth day contained negligible amounts of radioactivity. Most of the radioactivity was found in the glucosiduronate fractions of both urines, and only a small percentage of the injected dose was recovered in the sulfate extracts (Tables 50 and 59). There was a poor recovery of radioactivity (40-50%) from both urines after the hydrolysis procedure. One possible explanation that could account for the remainder of the radioactivity is the large error involved in counting crude urine samples. In some instances, late pregnancy urine usually contains a heavy sediment, thus, making it very difficult to obtain a homogeneous sample of the urine for counting.

Three metabolites were identified in the glucosiduronate fraction of the urine of each subject; no metabolites were identified in the sulfate extract from the urine of subject BB. The identified compounds, together with their final $^{3}H/^{14}C$ ratios and the radioactivity in each metabolite, expressed as a percentage of the injected dose, are shown in Table 66. Unlike the previous experiment, only one of the injected substrates, namely, 15α-hydroxyandrostenedione, was isolated from the urine of each subject; no 15α-hydroxydehydroisoandrosterone was detected in the urinary extracts. The isolated 15α-hydroxyandrostenedione contained both the

Table 66

Urinary Metabolites Isolated in Experiment 5 Following the Administration of ^{3}H-15α-Hydroxyandrostenedione and ^{14}C-15α-Hydroxydehydroisoandrosterone to Two Fetuses in Utero

Metabolite	Subject BB ($^{3}H/^{14}C$ = 4.7)			Subject EW ($^{3}H/^{14}C$ = 4.1)		
		% of Injected Dose			% of Injected Dose	
	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C
15α-Hydroxyandrostenedione	8.2	1.30	0.73	2.8	0.28	0.47
15α-Hydroxyestradiol	22.5	0.14	0.03	21.3	0.20	0.04
15α-Hydroxyestriol	9.4	0.26	0.13	16.3	1.05	0.31
15α-Hydroxydehydroisoandrosterone	-	-	-	-	-	-
Unknown	0.0*	0.00*	2.45*	0.0*	0.00*	1.43*

*These values were computed from the amount of radioactivity obtained after elution of the unknown metabolite from the last chromatogram.

^{3}H and ^{14}C labels, indicating a conversion of 15α-hydroxydehydroisoandrosterone to this metabolite. Although the low $^{3}H/^{14}C$ ratio (2.8) of the urinary 15α-hydroxyandrostenedione from subject EW indicates a large conversion of 15α-hydroxydehydroisoandrosterone to this metabolite, the actual percentage conversion was 0.47%, which is lower than that (0.73%) for subject BB.

An unknown metabolite was also isolated from the urines of both subjects in this experiment. It contained only the carbon-14 label and had the same chromatographic mobility on paper as the unknown metabolite isolated in the previous experiment. Also, it contained an insufficient amount of weight for physical and chemical identification.

The $^{3}H/^{14}C$ ratios (Table 66) of 15α-hydroxyestradiol and 15α-hydroxyestriol show that 15α-hydroxyandrostenedione is a better precursor of these estrogens than 15α-hydroxydehydroisoandrosterone when both substrates are injected directly into the fetus. Furthermore, on the basis of the percentage conversion of each substrate to the isolated 15α-hydroxyestrogens, it is obvious that considerably more 15α-hydroxyestriol than 15α-hydroxyestradiol is formed from these precursors.

Comparison of the percentage conversion (Table 66) of 15α-hydroxyandrostenedione to the urinary 15α-hydroxyestrogens isolated from subjects BB and EW with those obtained in the parallel study by YoungLai et al (46) reveals that somewhat smaller conversions were obtained in these studies. Their results showed that the percentage conversion of 15α-hydroxyandrostenedione to urinary 15α-hydroxyestradiol in two subjects was 0.15 and 0.88, whereas the conversion of this substrate to urinary 15α-hydroxyestriol was 0.39% and 1.49%. The lower values of the percentage

conversions just given for both 15α-hydroxyestrogens were obtained from the same experiment and the authors suggested that they may be due to the fact that the first voiding in that study was lost.

From the results just presented, one can visualize several pathways for the formation of 15α-hydroxylated estrogens from 15α-hydroxydehydroisoandrosterone in late pregnancy. A consideration of the $^{3}H/^{14}C$ ratios of the isolated urinary 15α-hydroxyandrostenedione and 15α-hydroxyestriol in Experiments 4 and 5 reveals that, with the exception of the data pertaining to subject EW, there is very little difference between the ratios of these two metabolites in each study. It was pointed out earlier that the majority of the total tritium and carbon-14 recovered from the urine of each of the four subjects investigated was excreted in the first day. Since the first voiding of subject EW was lost and since there is a possibility that the labeled urinary metabolites were excreted at different rates, it is justifiable to exclude the results of subject EW in the present interpretation of the $^{3}H/^{14}C$ ratios shown in Tables 65 and 66. In such a case, these ratios suggest that 15α-hydroxydehydroisoandrosterone is converted to 15α-hydroxyestriol via 15α-hydroxyandrostenedione. However, it is also possible that 15α-hydroxydehydroisoandrosterone may first be 16α-hydroxylated and converted to 15α,16α-dihydroxyandrostenedione prior to aromatization and the subsequent formation of 15α-hydroxyestriol.

The $^{3}H/^{14}C$ ratios of the isolated urinary 15α-hydroxyandrostenedione and 15α-hydroxyestradiol in each of the studies under consideration differ by a considerable amount. This finding suggests that the formation of 15α-hydroxyestradiol from 15α-hydroxydehydroisoandrosterone via 15α-hydroxyandrostenedione is a pathway of minor importance. An alternate

pathway may involve the conversion of 15α-hydroxydehydroisoandrosterone to 15α-hydroxyestradiol via androst-5-ene-3β,15α,17β-triol in analogy to the following biosynthetic sequence: 16α-hydroxydehydroisoandrosterone sulfate → androst-5-ene-3β,15α,17β-triol sulfate → estriol. Definite evidence that the latter biosynthetic sequence is operative during pregnancy was offered by YoungLai and Solomon (85).

From previous data (28,46) and the results presented in this thesis, it is obvious that the metabolic pathways leading to the formation of 15α-hydroxylated estrogens have not yet been fully clarified. Further studies must be carried out in order to elucidate these pathways. It is, however, clear that 15α-hydroxydehydroisoandrosterone is not an important precursor of 15α-hydroxyestriol as may be expected from our knowledge of the formation of estriol during late pregnancy.

SUMMARY AND CONCLUSIONS

Two different sets of experiments are described in this thesis. When labeled pregnenolone was injected into the umbilical vein of a Rhesus monkey with the fetus in utero, most of the radioactivity was found in an ether-soluble form. The substrate was recovered from all the fetal tissues and the placenta in sizable amounts. From the fetal heart, intestine, liver and from the placenta, it was possible to isolate 3β-hydroxy-5α-pregnan-20-one. Also, the conversion of pregnenolone to progesterone by the fetal liver was demonstrated. From this study, it was concluded that, unlike human pregnancy, pregnenolone is not metabolized to any extent by the fetal tissues and placenta of the Rhesus monkey.

The second set of experiments involved the synthesis of 15α-hydroxydehydroisoandrosterone and a study of its isolation and metabolism. Non-labeled 15α-hydroxydehydroisoandrosterone was synthesized chemically from 15α-hydroxyandrostenedione. This steroid was identified by its infrared and mass spectra and by elemental analysis. Further confirmation of the structure of 15α-hydroxydehydroisoandrosterone was obtained by converting it ot 15α-hydroxyandrostenedione enzymatically with 3β-hydroxysteroid dehydrogenase-Δ^5-isomerase. The diacetate of 15α-hydroxydehydroisoandrosterone was also prepared and it was identified by infrared, mass, NMR and elemental analyses. The overall yield of 15α-hydroxydehydroisoandrosterone from 15α-hydroxyandrostenedione was over 6%. Similarly, radiochemically pure ^{14}C-15α-hydroxydehydroisoandrosterone was prepared from ^{14}C-15α-hydroxyandrostenedione; the overall yield of the former compound was 8%.

Minute amounts of 15α-hydroxydehydroisoandrosterone were isolated from two different pools of human late pregnancy urine. Purified ^{14}C-15α-hydroxydehydroisoandrosterone was added to the urine as a recovery marker and the steroid was isolated and identified. Using the isotope derivative procedure, the amount of 15α-hydroxydehydroisoandrosterone excreted in the urine was found to range from 1.76 to 3.20 μg/day. This finding demonstrated that 15α-hydroxydehydroisoandrosterone is a normal excretory product in the third trimester of human pregnancy.

When a mixture of ^{3}H-15α-hydroxyandrostenedione and ^{14}C-15α-hydroxydehydroisoandrosterone was injected intravenously into each of two normal subjects in the third trimester of pregnancy, the following metabolites containing both labels were isolated from the glucosiduronate fraction of the urine of each subject: 15α-hydroxyandrostenedione, 15α-hydroxyestradiol and 15α-hydroxyestriol. Also, in both studies, a small percentage of the injected 15α-hydroxydehydroisoandrosterone was recovered from the urine and contained only the ^{14}C label. In addition, both sets of urine contained an unidentified metabolite which was derived solely from 15α-hydroxydehydroisoandrosterone and which accounted for a sizable portion of the excreted metabolites. From these studies, it was concluded that in the third trimester of human pregnancy 15α-hydroxydehydroisoandrosterone is a good precursor of urinary 15α-hydroxyandrostenedione, however, the latter compound appears to be a more efficient precursor of urinary 15α-hydroxyestradiol and 15α-hydroxyestriol than 15α-hydroxydehydroisoandrosterone.

Two studies were also performed using an experimental design in which a mixture of ^{3}H-15α-hydroxyandrostenedione and ^{14}C-15α-hydroxydehydroisoandrosterone was introduced into the fetus during transfusion

in utero for erythroblastosis fetalis. The metabolites isolated from the maternal urine in these studies were the same as those obtained in the previous experiment, with the exception of 15α-hydroxydehydroisoandrosterone. This compound could not be detected in the urine of both subjects and appeared to be completely metabolized by the fetus. The results of these studies served to strengthen the conclusions drawn from the previous experiment.

CLAIMS TO ORIGINAL RESEARCH

1. Except for the fetal liver, the fetal tissues and placenta of the Rhesus monkey did not metabolize pregnenolone to any extent. 3β-Hydroxy-5α-pregnan-20-one was isolated from the fetal heart, intestine, liver and placenta of the Rhesus; pregnenolone can be converted to progesterone by the fetal liver. The pregnant Rhesus monkey cannot serve as a model for steroid metabolism in human pregnancy.
2. Non-labeled and labeled 15α-hydroxydehydroisoandrosterone was synthesized chemically from 15α-hydroxyandrostenedione.
3. 15α-Hydroxydehydroisoandrosterone was shown to be present in human urine during the third trimester of pregnancy.
4. 15α-Hydroxydehydroisoandrosterone was converted to urinary 15α-hydroxyandrostenedione, 15α-hydroxyestradiol and 15α-hydroxyestriol, following the injection of the substrate into a subject in the third trimester of pregnancy or into the fetal compartment.
5. 15α-Hydroxyandrostenedione was a more efficient precursor of urinary 15α-hydroxyestradiol and 15α-hydroxyestriol than 15α-hydroxydehydroisoandrosterone.

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