A METHOD FOR THE ANALYSIS OF SPECIFIC URINARY 3α-HYDROXY-Δ^5-STEROIDS AND SOME APPLICATIONS IN CLINICAL MEDICINE

by

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A THESIS
Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Master of Science
April, 1965

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Committee)

ACKNOWLEDGEMENTS:

I gratefully acknowledge the instruction, advice and encouragement given by Doctor A. Colás, Professor in charge of this thesis. I appreciate the encouragement given by Drs. H. J. Tatum and R. C. Benson, director and co-director, respectively, for the Cancer Research Training Program in the Department of Obstetrics and Gynecology.

This study was supported by Grant No. CA-5081 from the United States Public Health Service.

I appreciate the kind cooperation of all persons who submitted specimens for analysis, the assistance of physicians and nurses who participated in the collection of specimens, the technical assistance by Mr. Gus Ebro and Mr. Fabio Herrera, and secretarial help given by Evelyn Sacressen.

NAMES OF COMPOUNDS USED IN THE TEXT

Trivial and Systematic Names

3β-OH-Δ^5-steroids - 3β-hydroxy-Δ^5-steroids

cholesterol - cholest-5-en-3β-ol
17α,20α-OH-cholesterol - cholest-5-ene, 3β, 17α, 20α-triol
20α,22-diOH-cholesterol - cholest-5-ene, 3β, 20α, 22-triol

pregnenolone - 3β-hydroxypregn-5-en-20-one
16α-OH-pregnenolone - 3β,16α-dihydroxypregn-5-en-20-one
17α-OH-pregnenolone - 3β,17α-dihydroxypregn-5-en-20-one
21-OH-pregnenolone - 3β,21-dihydroxypregn-5-en-20-one
16α,17α-diOH-pregnenolone - 3β,16α,17α-trihydroxypregn-5-en-20-one
pregnenediol - pregn-5-ene-3β,20α-diol
20β-pregnenediol - pregn-5-ene-3β,20β-diol
16α-OH-pregnenediol - pregn-5-ene-3β,16α,20α-triol
pregnenetriol - pregn-5-ene-3β,17α,20α-triol
20β-pregnenetriol - pregn-5-ene-3β,17α,20β-triol
pregnenetetrol - pregn-5-ene-3β,17α,20β,21-tetrol
progesterone - pregn-4-ene-3,17-dione
17α-OH-progesterone - 17α-hydroxypregn-4-ene-3,17-dione
cortisol - 11β,17α,21-trihydroxypregn-4-ene-3,20-dione

dehydroepiandrosterone, DHA or
androstenolone - 3β-hydroxyandrost-5-en-17-one
androstenediol - androst-5-ene-3β,17β-diol
7α-OH-DHA - 3β,7α-dihydroxyandrost-5-en-17-one
7-keto-DHA - 3β-hydroxyandrost-5-ene-7,17-dione
16α-OH-DHA - 3β,16α-dihydroxyandrost-5-en-17-one
7α,16α-diOH-DHA - 3β,7α,16α-trihydroxyandrost-5-en-17-one
16α-OH-7-keto DHA - 3β,16α-dihydroxyandrost-5-ene-7,17-dione
16-ketoandrostenediol - 3β,17β-dihydroxyandrost-5-en-16-one
androstenetriol - androst-5-ene-3β,16α,17β-triol
16β-androstenetriol - androst-5-ene-3β,16β,17β-triol
androsterone - 3α-hydroxy-5α-androstan-17-one
etiocholanolone - 3α-hydroxy-5β-androstan-17-one
androstenedione - androst-4-ene-3,17-dione
11β-OH-androstenedione - 11β-hydroxyandrost-4-ene-3,17-dione
testosterone - 17β-hydroxyandrost-4-en-3-one
testosterone acetate - 17β-acetoxyandrost-4-en-3-one

estrone - 3-hydroxyestra-1,3,5(10) - trien-17-one
estradiol - estra-1,3,5(10)-triene-3,17β-diol
estriol - estra-1,3,5(10)-triene-3,16α,17β-triol
16α-OH-estrone-3,16α-dihydroxyestra-1,3,5(10)-trien-17-one
16-keto-estradiol-3,17β-dihydroxyestra-1,3,5(10)-trien-16-one
16-epiestriol - estra-1,3,5(10)-triene-3,16β,17β-triol

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I. INTRODUCTION

A. STATEMENT OF THE PROBLEM.

In studies of biosynthetic and metabolic sequences, one would like to know the precise nature and quantity of compounds that perform a specific metabolic activity in the cells and the mechanisms and controls of the biochemical changes. Since this information has rarely been obtained, most physiologic concepts are based upon secretory and excretory data obtained *in vivo* from healthy, diseased or experimentally altered subjects, and upon transformations *in vitro* by their tissues. This basis has been necessary particularly in the case of the steroid hormones which are present in very minute quantities within the organism. In addition, progress has been delayed by the necessity to use complex methods to isolate steroids from the other lipids in biological materials and the rigorous identification procedures that must be applied to recognize the small chemical differences existent among grossly similar molecules. The problem undertaken in this study is an examination of the excretion rates of the major urinary 3β-hydroxy-Δ^5-steroids in normal and diseased human subjects. An evaluation of several available techniques for this estimation is of fundamental importance and is included. This study is one part of the systematic analysis of the significance of this group of steroid hormones in human metabolism.

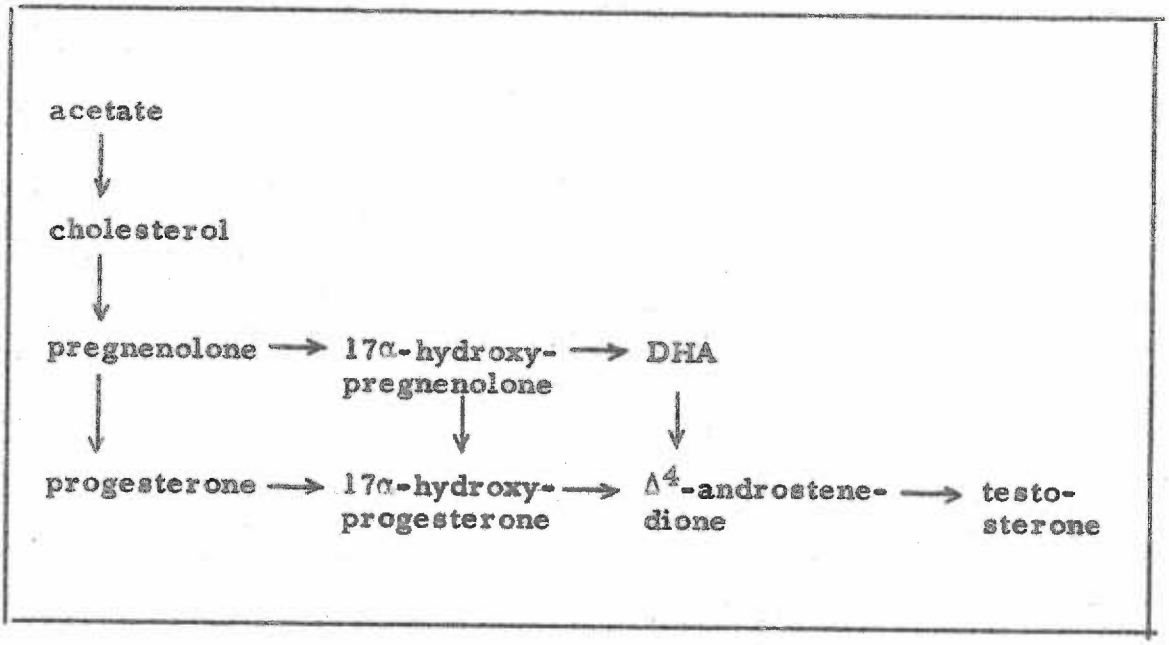
B. ISOLATED AND IDENTIFIED URINARY METABOLITES.

More than three decades have elapsed since the first urinary steroids were identified. Since then, an enormous amount of physiological knowledge about the significance of the various steroid hormones has accumulated. Strides in methodology are mainly responsible for the rapid advances that have been possible in elucidating the chemical identity of compounds that comprise biosynthetic and metab olic pathways. Unlike the adrenocorticosteroids, the identification and quantitative estimations of the androgen sequences are very recent accomplishments. However, as a result of many experiments, two major but different biosynthetic sequences for androgens common to steroidogenic tissues have been established (96). (See Figure No. 1) Pregnenolone, characterized by the 3β-ol-Δ^5-configuration in rings A and B, occupies a pivotal position in the scheme. The same configuration is retained in compounds that comprise one of the sequences for the biosynthesis of androgens (pregnenolone ⟶ DHA). The isolation of nearly twenty different 3β-hydroxy-Δ^5-steroids from urine of patients and normal human subjects emphasizes the probability of a significant role of these compounds in steroid metabolism.

In 1934, Butenandt and Dannenbaum isolated and identified dehydroepiandrosterone (DHA) from a 1.2 kg. fraction extracted from 143,000 liters of male urine (17). A decade later, Marrian and Butler reported the isolation of androstenetriol from urine (78)

FIGURE NO. 1. Principal biosynthetic sequences for androgens.

TABLE NO. 1. Urinary 3β-OH-Δ^5-steroids excreted by a boy with adrenal cancer [Hirschmanns (52, 56)].



Steroid	Mg./l.	Year Reported
androstenetriol	400.0	1943
androstenediol	7.7	1945
pregnenediol	35.0	1945
17α-OH-pregnenolone	11.7	1947
16α-OH-pregnenediol	6.0	1950
pregnenetriol	1.3	1950

by men and pregnant and nonpregnant women. The rate of excretion was about 0.1 mg./24 hours. About the same time, Hirschmann found about 400 mg. per liter of the same compound in the urine from a 7-year-old boy with adrenal cancer (52-56). Hirschmann advanced the idea that androstenetriol was a metabolic product of DHA similar to the metabolic conversion of estrone to estriol. That same boy's urine was the source for five other previously unrecognized, naturally-occurring 3β-hydroxy-Δ^5-steroids that the Hirschmanns reported during the following seven years (52-56). The compounds and quantities they isolated are listed in Table No. 1.

In renewed efforts by the Edinburgh group, Fotherby, et al.(33) reported in 1957 the isolation of 16α-OH-DHA in urine from normal males. The significance of this compound is that a ring-D α-ketol is a probable intermediate in the "hydration" sequence leading from dehydroepiandrosterone to androstenetriol; the phenolic analogs had already been demonstrated (12). An alternate order of the sequence was suggested by Fotherby (34) after he found the ring-D primary alcohol, androstenediol in urine from normal men. He also found the 16β-isomer of androstenetriol and showed that pregnenetriol, found initially by Hirschmann in the urine of cancer patients, was also present in normal urine. In 1959, Fotherby (35) reported the isolation of 16α-pregnenediol, again in urine from normal subjects.

In 1958, Gallagher (41) reported that patients with adrenal cancer excreted 7-keto-DHA in amounts of 2 - 12 mg./24 h. This

compound had been isolated and characterized earlier by Fukushima, et al. (37, 38) from normal subjects and patients with adrenal disease. Kappas, et al. (62), working in the same laboratory, mentioned that 7-keto-DHA was nearly always present in the urine of normal women but he reported no excretion values. Also from the Sloan-Kettering Institute for Cancer Research, Okada, et al. (87), reported that five 3β-hydroxy-Δ^5-steroids were isolated and characterized (but not all were quantitated) in urine from another patient with adrenal cancer. Three previously recognized urinary steroids, pregnenetriol, 16α-OH-DHA and 7α-OH-DHA [the latter had been found in urine earlier after administration of a precursor (59)], were present in mg./24 h. amounts. Two newly recognized urinary products, 16α-OH-7-keto-DHA, 7α,16α-diOH-DHA were present in unreported amounts in this patient. In another cancer patient's urine, Fukushima, et al. (38) found 7-keto-DHA, 20α-pregnenetriol and 20β-pregnenetriol, all in mg./24 h. amounts.

In 1962, Bongiovanni, et al. (8, 9, 10), also reported an incompletely identified urinary product that they tentatively labelled 16α-OH-7-keto-DHA in cases of one type of adrenogenital syndrome. They did, however, identify and measure a new urinary steroid, pregnenetetrol, in those cases and in pools of urine from normal newborn infants. This is the first tetrahydroxy-Δ^5-steroid in the C_{19} or C_{21} classes to be found in urine. They also measured mg. amounts of another previously unrecognized urinary steroid, 16α-OH-pregnenolone in urine of the patients but none from normal infants. Four other urinary 3β-OH-Δ^5-steroids were present and measured. Rey-

nolds (92) confirmed the finding of 16α-OH-pregnenolone in the patients with adrenal hyperplasia and also found it in urine from a newborn patient who was endocrinologically normal. He (93) isolated another C-16-oxygenated metabolite, 16-ketoandrostenediol from the urine of newborn infants and has found the excretion rate for a mixture of roughly equal parts of 16-ketoandrostenediol and 16α-OH DHA to range between 0.1 and 0.9 mg. per 24 hours in normal infants up to 5 months old and 0.3 - 1.4 mg. per 24 hours from a child with adrenal hyperplasia.

In 1962, Pasqualini and Jayle (90) identified 21-hydroxypregnenolone as the disulfate in urine of normal but ACTH stimulated pregnant and nonpregnant subjects in less than 100 μg./l. amounts. They stated that Crepy (personal communication) had noted the same fraction earlier in urines from normal and pathological subjects. Only a year later, Cathro, et al. (21), declared that they had provisionally identified the same steroid in the urine from infants, puerperal and nonpregnant women in amounts of 28, 120 and 63 μg./24 h., respectively.

The significance of these eighteen 3β-OH-Δ^5-steroids in urine is mostly unknown. Their excretion in urine is compatible with normal or pathological function of the adrenal gland and the trend of excretion values is higher where neoplastic or hyperplastic adrenal tissue exists. (See Table No. 2) Their significance is certainly not limited to pathological states since measurable quantities of fifteen of the eighteen compounds were present in urine of normal subjects.

TABLE 2. Urinary 3$^{\alpha}$-OH-Δ^5-steroids: Range of values (mg./24 h. unless indicated) from normal subjects and others with adrenal or ovarian disease taken from a literature survey.

Steroid	Reference	Subject		
		Normal	Adrenal Disease	Ovarian Disease
16α-OH-pregnenolone	(92, 10,)	0 - 0.81	3.0 - 8.1	—
17α-OH-pregnenolone	(10, 54,) (, 8,)	0 - 1.2	0.9 - 11.7	—
21α-OH-pregnenolone	(21, ,)	0.028 - 0.120	—	—
pregnenediol	(112, 97, 112)	0.08 - 0.5	—	0 - 0.15
16α-OH-pregnenediol	(35, 55,)	0.008/1.	6.0/1.	—
pregnentriol	(112, 88, 27) (, , 104)	0.08 - 1.1	0.07 - 66	0.2 - 1.0
20β-pregnenetriol	(, 38,)	—	2.0	—
pregnenetetrol	(10, 9,)	0.2	0 - 5	—
DHA	(36, 97, 112) (112, , 95)	0 - 4.5	2.3 - 702	0.4 - 9.58
androstenediol	(112, 97, 112)	0.5	0 - 24.5/1.	0 - 0.05
7α-OH-DHA	(, 87,)	—	present	—
7-keto-DHA	(62, 38,) (37, 41,)	present	3.1 - 12/1.	—
16α-OH-DHA	(34, 8,) (33, 10,)	0.05 - 0.15	0.3 - 3.5	—
7α, 16α-diOH-DHA	(, 87,)	—	present	—
16α-OH-7-keto-DHA	(, 87,)	—	present	—
16-ketoandrostenediol + 16α-OH-DHA	(93, 93,)	0.1 - 0.9	0.3 - 1.4	—
androstenetriol	(78, 52,) (34, ,)	0.04/1. - 0.1	400/1.	—
16β-androstenetriol	(34, ,)	0.036	—	—

C. BIOSYNTHESIS AND METABOLISM.

Cholesterol as a Precursor of Steroids

The idea that cholesterol could be the precursor of steroid hormones developed as the chemical structures of newly-identified hormones from the adrenal gland, testis, and ovary were found to be cyclopentanoperhydrophenanthrene derivatives (51). Cholesterol was first established as a precursor by transformations in vivo of isotopically labeled cholesterol into urinary pregnanediol and estrone (6, 109). The numerous chemical transformations required in the biogenesis of cholesterol will not be reviewed here but they can be summarized in three principal steps (7):

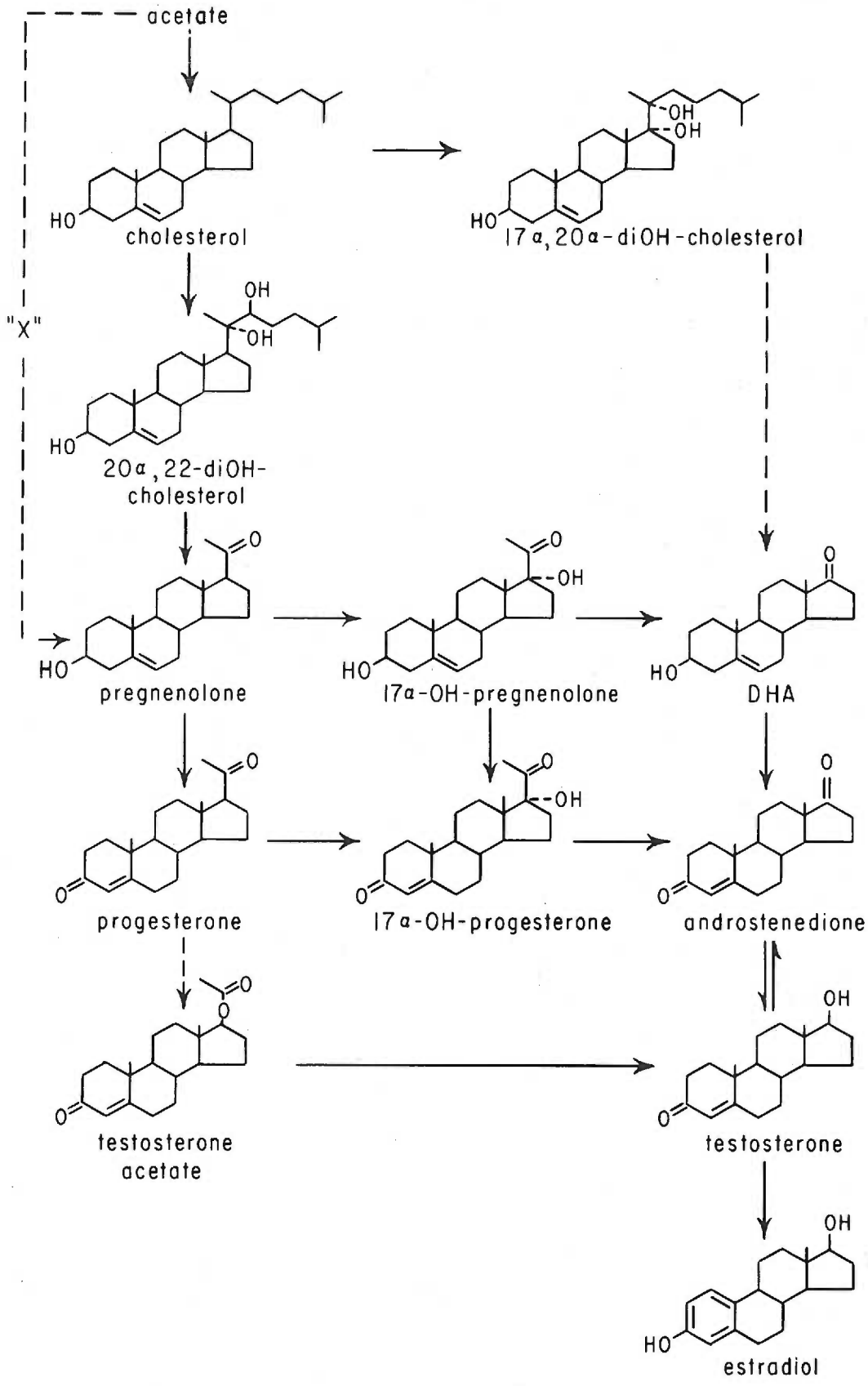
(1) condensation of 18 acetate molecules to form squalene,

(2) cyclization of squalene to form lanosterol, and

(3) demethylation of lanosterol to produce cholesterol.

The conversion of cholesterol to the specific steroid compounds that characterize the physiological secretion product(s) of the adrenal, testis, and ovary has been amply demonstrated (29). In each gland, the evidence is compatible with the biosynthetic sequences outlined in Fig. 2 where pregnenolone is a pivotal compound from which C_{21} corticosteroids, progestins or C_{19} androgens and thereafter, C_{18} estrogens can be derived (28, 46). The mechanism for splitting off the side chain of cholesterol to form pregnenolone appears to involve at least two hydroxylation reactions at C-20 and C-22 and the action of a side-chain-splitting enzyme system (desmolase) to sever the bond between C-21 and C-22. Such a desmolase

FIGURE NO. 2. Alternative routes of androgen biosynthesis. Another route, not depicted in the figure, consists of C-3 sulfate esters of the 3β-OH-Δ^5-compounds. The dashed lines represent unproven sequences and "x" represents unknown compounds.


acetate
cholesterol
17α,20α-diOH-cholesterol
"X"
20α,22-diOH-cholesterol
pregnenolone
17α-OH-pregnenolone
DHA
progesterone
17α-OH-progesterone
androstenedione
testosterone acetate
testosterone
estradiol

has been described in adrenal and gonadal tissue (51). A proposed intermediate compound, 20α, 22-dihydroxycholesterol, has yielded pregnenolone in adrenal homogenate incubations (100) and cholesterol was transformed to pregnenolone by bovine adrenal mitochondria (67).

Other Precursors of C_{19} Steroids

Other biosynthetic sequences have been postulated for the conversion of cholesterol and even noncholesterol precursors directly to C_{19}-steroids (29, 51, 69). One involved in the direct biosynthesis of 3β-OH-Δ^5-C_{19} steroids requires a side-chain scission between C-17 and C-20 which results in the formation of DHA without passing through any C_{21} compound. Indirect evidence given in support of this concept is a 25 percent variation of the ratio of isotopic activities of two isotopes when cholesterol and pregnenolone, each with a different isotopic label were incubated with adrenal tissue from a patient with Cushing's Syndrome (49). However, workers have failed recently to find evidence for this pathway in incubations of rat adrenal or testis homogenates (101). Another sequence to testosterone from cholesterol via pregnenolone ⟶ progesterone includes the possibility of testosterone acetate serving as the intermediate precursor of testosterone. Definitive experiments are necessary to establish this sequence. The possibility of noncholesterol precursors for androgens (labeled "X" in Fig. 2) was first suggested by higher specific activities of cortisol than of cholesterol after radioactive acetate was administered to human subjects. However, increased knowledge of 'cholesterol pools' allows alternative conclusions from those data.

Finally, recent evidence has accumulated showing that the sulfate esters of steroids are secreted by steroidogenic tissues and can serve with good efficiency as precursor substrates in studies in vitro for production of androgens (20). Finding cholesterol-sulfate in bovine adrenal tissue extracts (30) and circulating in human plasma (31) and demonstration of metabolism in vivo in adrenal cancer patients (19) of pregnenolone-sulfate ⟶ 17α-OH-pregnenolone-sulfate ⟶ DHA-sulfate support the conclusion that DHA-sulfate measured in normal adrenal venous effluent is a secretory product (110). DHA-sulfate has been demonstrated as an effective precursor for testosterone and androstenedione in canine testis and ovary (1) and for estrogens in nonpregnant females (108) and during pregnancy (3). These findings substantiate the concept that both free and sulfated steroids are actively metabolized in mammals and specifically in humans.

Differences between C_{21} and C_{19} Steroid Metabolism

The established major routes for the biosynthesis of C_{21} steroids are based upon the enzymic scission of isocaproic acid off the cholesterol molecule. The question of an obligatory role for cholesterol in steroid biosynthesis is not entirely resolved. It is a good substrate in vivo, but not in vitro, whereas acetate is effective in both types of systems. Intermediate compounds between acetate and cholesterol have not been adequately assessed as steroid precursors although mevalonic acid yields DHA in good amounts (29) and lanosterol yields testosterone (44). C_{21} steroid compounds may

serve as the source for the C_{21} adrenocorticosteroids (pregnenolone ⟶ progesterone ⟶ cortisol) and the C_{19} androgens (pregnenolone ⟶ progesterone ⟶ androstenedione) or exhibit typical biologic activities of their own (pregnenolone ⟶ progesterone). In each pathway, transformation of the 3β-OH-Δ^5-steroids to 3-keto-Δ^4-intermediate compounds is required to confer maximum biological activity.

In contrast to C_{21} hormones, which appear to require pregnenolone as an intermediate, potent C_{19} androgens may also be derived from biosynthetic sequences in which no C_{21} precursors participate and in which the immediate precursors retain the 3β-ol-Δ^5-configuration in rings A and B (DHA ⟶ androstenedione). The proximity in the biosynthetic schemes of the 3β-ol-Δ^5-steroids to potent androgens has stimulated great interest in the metabolism and significance of this group of compounds. Biosynthesis in two parallel schemes via the 3-keto-Δ^4- or the 3β-OH-Δ^5-series allows the possibility of control mechanisms for androgen secretion, but the relative quantitative contribution of either sequence is not known. The obligatory precursor role of androgens in the biosynthesis of C_{18} estrogens seems well established (12), but the significance, if any, of the large amounts of urinary androgen metabolites compared to the small quantities (μg.) of urinary estrogens is not apparent.

Transformations In Vivo and In Vitro

Metabolic studies in vivo of the transformation of pregnenolone and 17α-OH-pregnenolone to progesterone, DHA, androstene-

dione and testosterone have been confirmed repeatedly (29). The enzyme systems, 3β-ol hydroxysteroid dehydrogenase and Δ^5-Δ^4-steroid isomerase, which transform the Δ^5-substrates into the Δ^4-compounds, are present in adrenal, gonadal and placental tissues. Hydroxylation systems and reductases compete for the Δ^5-substrates in adrenal, gonadal, liver, kidney and/or placental tissues.

Isolation of the major urinary metabolites after intramuscular administration of DHA-acetate to a man with postsurgical pituitary insufficiency led to some of the earliest data about DHA metabolism (79). After over 1 gm. of DHA-acetate was injected, 130 mg. androsterone, 73 mg. etiocholanolone, 79 mg. DHA and 6.5 mg. of androstenediol were recovered from urine. From two patients with adrenal insufficiency, no DHA was normally excreted but after administration of DHA, small quantities of androstenediol or androstenetriol were measured (80). These findings were corroborated by results from incubations of rabbit and guinea pig liver where transformations of DHA into androstenediol and androstenetriol were abundant. Oral administration of androstenediol to a human subject resulted in the recovery of 3.4 percent as DHA, 1 percent androsterone and 3 percent as etiocholanolone derivatives (106). In other investigations of urinary metabolites after DHA administration to humans, 7α-OH-DHA and 16α-OH-DHA were recovered (33, 98, 102, 103). These were considered catabolic metabolites. Further evidence that 3β-OH-Δ^5-steroids contribute to androgens comes from incubations of adrenal cancer and placental tissues; androstenediol-17α-^{3}H was converted directly to testosterone-17α-^{3}H with 15-50 percent yields, respectively (4).

The canine testis and liver appear to utilize this sequence selectively during perfusion experiments (50). Also, evidence from studies in vivo suggests that the preovulatory peak of estrogen excretion during the menstrual cycle of normal females is achieved by the 3β-ol-Δ^5-steroid pathway (18). However in other studies DHA biosynthesis in vitro from acetate was more abundant in the luteal phase in ovaries from breast cancer and other gynecological patients. No trophic hormones had been added to the latter incubation mixtures (32). Plasma and urinary concentrations of DHA have been noted to vary cyclically (14).

Detection of androgen precursors in adrenal vein blood in concentrations higher than in peripheral blood exhibits prima facia evidence that the adrenal gland secretes those compounds. $C_{19}O_2$ compounds measured consistently in normal subjects are DHA, DHA-sulfate and androstenedione (110). Androstenediol, 17α-OH-pregnenolone and testosterone are sometimes found in small amounts. These data confirmed the concept that androgens secreted by the adrenal gland represented a large share of the body's androgen synthesis. Until 1961, DHA was thought to be exclusively an adrenal androgen (81), however, it was isolated from testicular (bovine) tissue (29) and secreted in mg. quantities during adrenal suppression in normal females, ostensibly from ovaries (73). The latter estimations were made using a double isotopic dilution technique which also disclosed a simultaneous secretion of testosterone and androstenedione. The major urinary excretion products of "normal" androgen metabolism are the saturated C_{19} compounds, androsterone and etiocholanolone, and un-

changed DHA in that order of decreasing quantities. These compounds comprise the majority of the neutral 17-ketosteroids, (17-KS), a popular measurement available in many clinical laboratories. The secretory and excretory products have the relationship shown in Figure No. 3 (72, 79, 81).

Chemical Relationships of 3β-OH-Δ^5-Steroids

Based upon the preceeding biosynthetic and metabolic sequences, the 3β-OH-Δ^5-steroids that have been identified in urine (excluding cholesterol, pregnenolone and 20β-pregnenediol in the figure) are depicted in Figure No. 4 in alignment according to chemical structure. Alternative transformations of the pivotal compounds, pregnenolone and DHA, are reduction of ketones, hydroxylations or dehydrogenation to 3-keto-Δ^4-steroids. A model for the interrelationship of Δ^5-compounds had been worked out for steroid estrogens shown in a simplified scheme in Figure No. 5 (12). According to present concepts, the oxido-reductions at ring D (shown according to functional classes of compounds in Figure No. 6), characterise the chemical alterations that differentiate the physiologically active androgens or estrogens.

The range of urinary values reported for each compound from normal subjects, or patients with adrenal or ovarian disease, are listed in Table No. 2. Inspection of the table demonstrates that the quantitatively important transformations of C_{21} compounds are 17α-hydroxylation and/or reduction of the C-20 ketone and among C_{19} compounds, C-17 ketone reduction and/or 16α-hydroxylation.

FIGURE NO. 3. Relation of Secretory and Excretory Products of Androgen Metabolism.

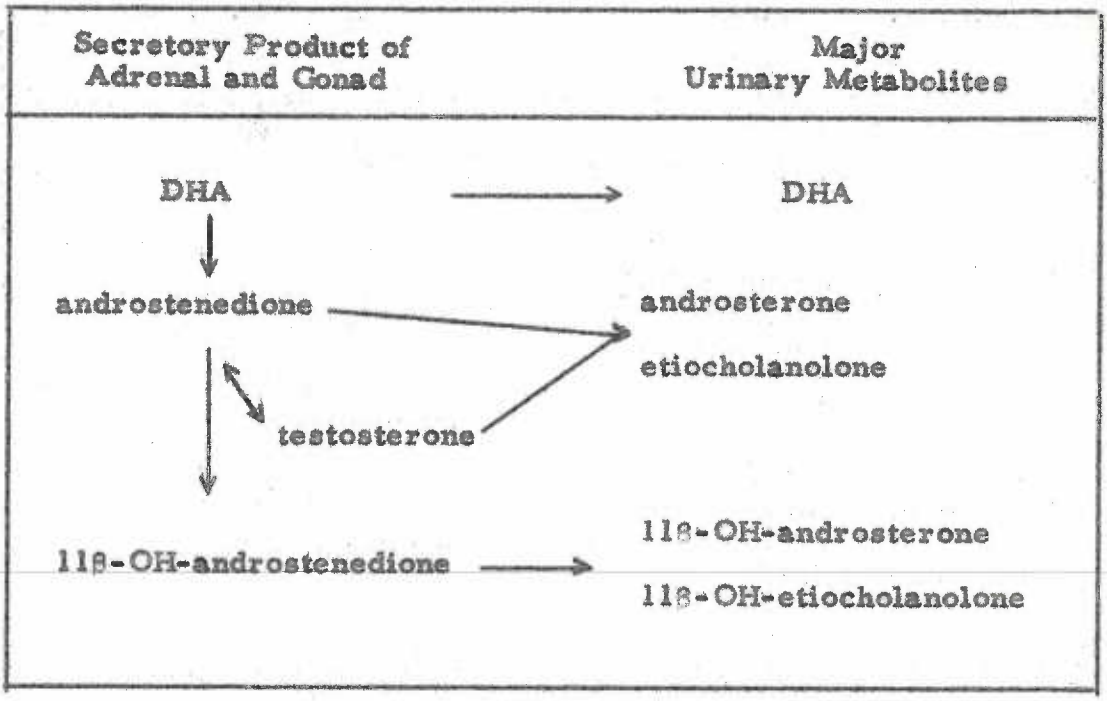

Secretory Product of Adrenal and Gonad
Major Urinary Metabolites
DHA
DHA
androstenedione
androsterone
etiocholanolone
testosterone
11β-OH-androstenedione
11β-OH-androsterone
11β-OH-etiocholanolone

FIGURE NO. 4. Biosynthetic and metabolic sequences of 3β-OH-Δ^5-steroids based upon the chemical structure of urinary metabolites. The compounds underlined are present in urine from normal subjects. (Cholesterol, pregnenolone and 20β-pregnenediol have not been found in urine.) Heavy lines demonstrate chemical relationships valid for steroid estrogens. Dotted lines indicate alternate transformations to 3-keto-Δ^4-steroids.

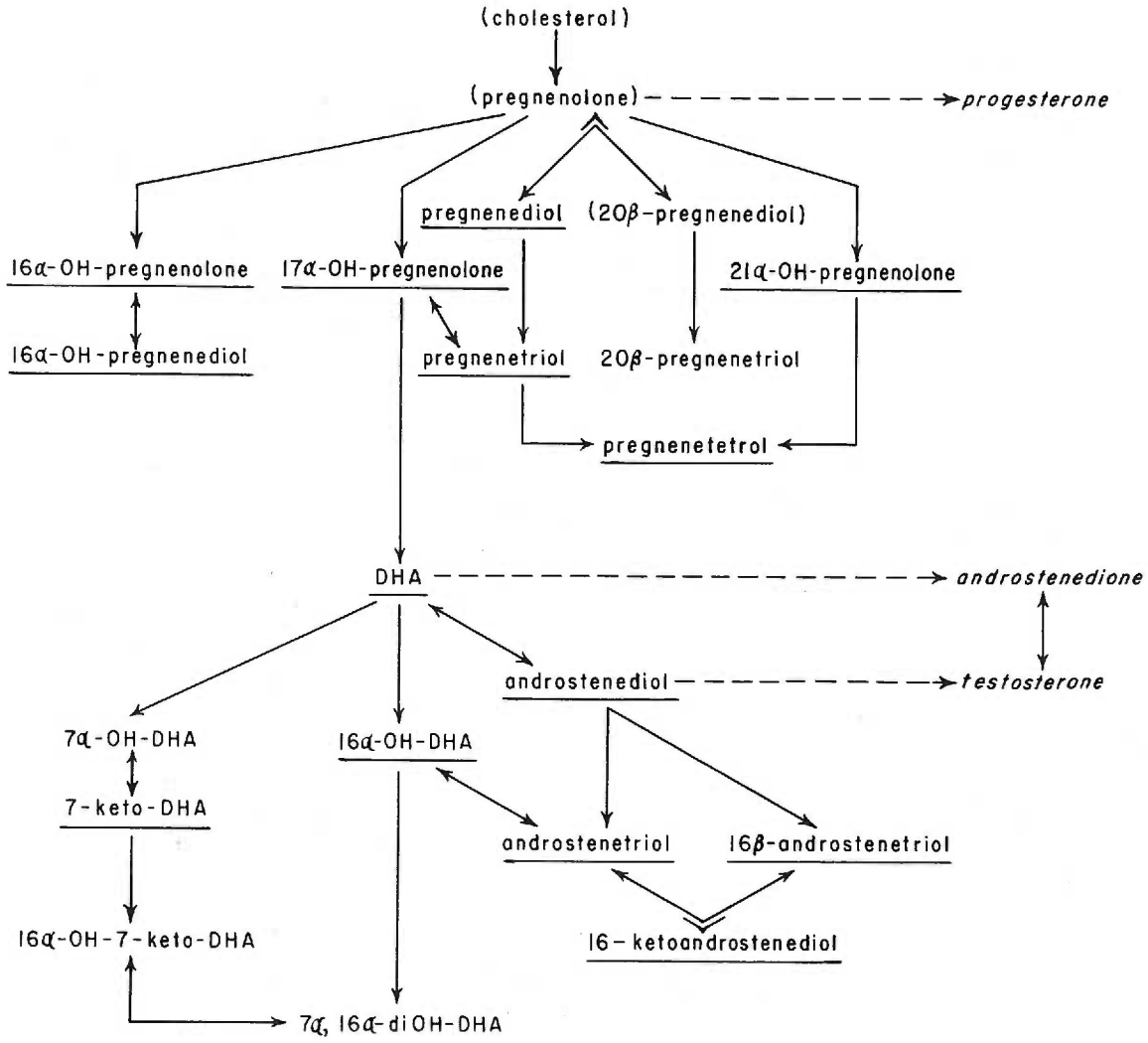
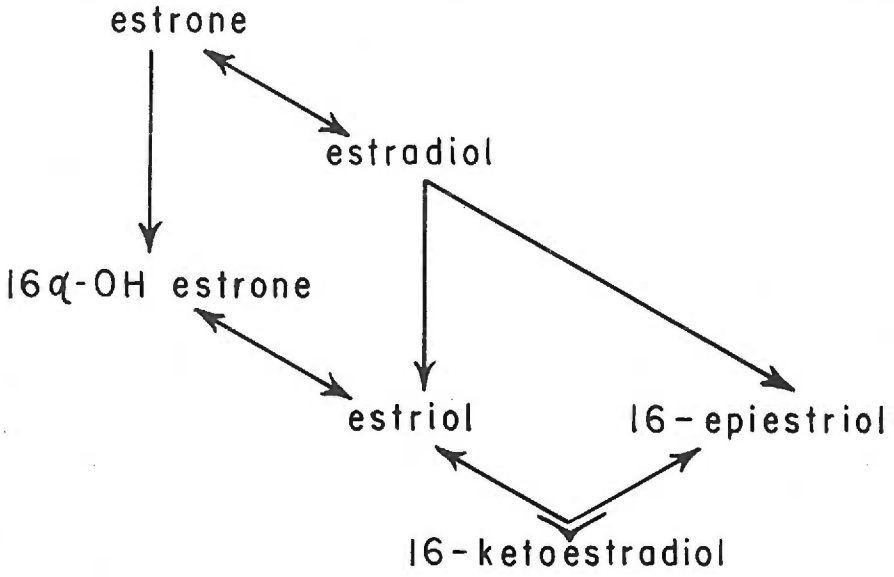
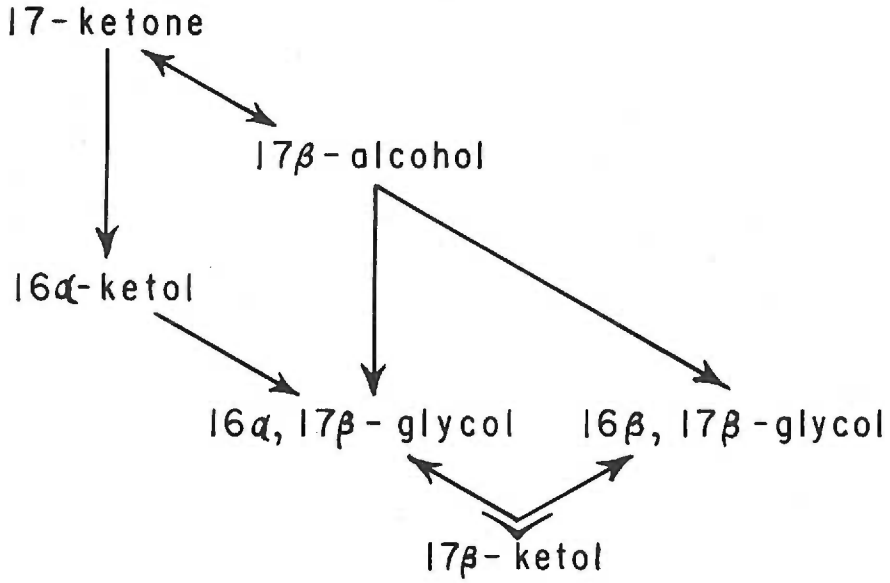

(cholesterol)
(pregnenolone)
progesterone
pregnenediol
(20β-pregnenediol)
16α-OH-pregnenolone
17α-OH-pregnenolone
21α-OH-pregnenolone
16α-OH-pregnenediol
pregnenetriol
20β-pregnenetriol
pregnenetetrol
DHA
androstenedione
androstenediol
testosterone
7α-OH-DHA
16α-OH-DHA
7-keto-DHA
androstenetriol
16β-androstenetriol
16α-OH-7-keto-DHA
16-ketoandrostenediol
7α, 16α-diOH-DHA

FIGURE NO. 5. Metabolic sequences of steroid estrogens based upon the chemical identity of urinary metabolites.

FIGURE NO. 6. Functional transformations at ring-D in steroid hormones.


estrone
estradiol
16α-OH estrone
estriol
16-epiestriol
16-ketoestradiol


17-ketone
17β-alcohol
16α-ketol
16α, 17β-glycol
16β, 17β-glycol
17β-ketol

Metabolism in Hirsutism and Virilism

As newer techniques for steroid analysis become available, investigations of patients with various endocrinopathies become widespread. Females who manifest hirsutism (masculine hair distribution), defeminization (cessation of menses, partial involution of primary and secondary sex characteristics) or virilism (partial sex reversal; clitoral hypertrophy, voice changes, etc.) were ideal subjects for investigation. Total urinary 17-ketosteroids which comprised the major urinary products of androgen metabolism, were observed to vary widely and inconsistently with the degree of masculinization (14). Fractionation of the 17-ketosteroids (17-KS) disclosed that the evaluation of the total quantity was often due to a disproportionate increase in DHA excretion, especially when adrenal gland neo- or hyperplasia caused the masculinizing symptoms. Elevations of the 11β-hydroxylated metabolites also correlated with adrenal disease. Ovarian pathologic alterations also proved to be capable of the disproportionate secretion of DHA but as with adrenal disease, biochemical data and clinical findings correlated poorly; some patients with regular menses and little or no abnormal hair growth had high 17-KS titers and vice versa (47). In addition, subsequent development of polycystic ovaries in some patients with primary adrenal disease has added confusion to the diagnosis of primary ovarian disease.

Experiments of two general types have been carried out in efforts to further elucidate the metabolic patterns of hirsutism: dynamic tests wherein glandular stimulation or suppression by administration of exogenous provocators [e.g. ACTH (adrenocorticotrophic

hormone) or cortisol or its analogs] was carried out simultaneously as urinary metabolites were sampled, and in another direction, detailed biochemical procedures in search of metabolites which might exhibit a more precise correlation with the degree of symptom expression. The dynamic tests devised to distinguish between adrenal and ovarian contributions to steroid metabolism have not offered a reliable diagnostic index (47, 48, 75, 76). As a result of detailed procedures devised to measure other steroids related to androgen metabolism, additional emphasis centered upon the 3β-OH-Δ^5-steroids. The amount of 17α-OH-pregnenolone and DHA extractable from polycystic ovarian tissue was greatly increased if pituitary trophic hormones (follicle stimulating hormone; FSH) had been administered(77). Androgen metabolism in incubations of pathologic ovaries including arrhenoblastoma, granulosa cell tumor and polycystic tissues selectively involved the 3β-OH-Δ^5-intermediates (63, 64, 65). In adrenal carcinoma patients, urinary excretion of pregnenetriol, pregnenediol, androstenediol and DHA varied widely and was greater than in normal subjects (112). The only correlation possible was an inverse relationship between pregnenetriol and DHA concentrations in urine. The amounts of pregnenediol and pregnenetriol were similar in normal females and those with polycystic ovaries but DHA was higher in the latter and two of the four patients with endocrinopathies excreted small amounts of androstenediol. Using different methods, other investigators found that pregnenetriol was nearly always present in urine from patients with polycystic ovaries but only in a few normal females (104). These studies of 3β-OH-Δ^5-compounds failed to iden-

tify a unique metabolite, or significant differences of urinary metabolites excreted in androgenic syndromes, but they confirmed some role for this group of steroids in androgen metabolism. A list of the amounts of the various 3β-OH-Δ^5-steroids found in various subjects is given in Table No. 3. (See footnote) In other investigations, undertaken more recently than the present study, measurements of plasma or urinary testosterone have demonstrated that elevated concentrations are present in some females who have masculinizing symptoms (40, 99). A definite sex difference and decreased concentrations with aging in males have also been observed. Since 1962, measurements of plasma and urinary testosterone concentrations have been possible. Values from normal males are higher than those from normal females. Some females with masculinizing symptoms have testosterone concentrations as high or higher than those found from normal men.

The truism that urinary steroids represent the net qualitative and quantitative result of biosynthetic rates in all steroidogenic tissues, a multitude of metabolic rates in the same and other tissues, and excretory rates might prompt the prediction that attempts to elucidate metabolic pathways from urinary studies are futile. Yet most of the data about steroid metabolism in vivo has accumulated as a result of urine quantitation. It is perhaps obvious that the simultaneous determination

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For most of the compounds listed in Table No. 3, an effort has been made to include all of the values reported in the literature. For other compounds, values have been selected that represent the scope of numerous, often similar, reports. Emphasis for the selection was placed on recent determinations in related conditions and on comparable methodology.

TABLE NO. 3. Urinary 3β-OH-Δ^5-steroids: List of values (mg./24 h. unless indicated) from different kinds of subjects taken from a literature survey.

Steroid	Amount	Subject	Reference
16α-OH-pregnenolone	0.21 - 0.81	newborn infant	(92)
	3.7 - 8.1	adrenal hyperplasia	(92)
	3 - 4.5	adrenal hyperplasia	(10)
17α-OH-pregnenolone	11.7/1.	adrenal tumor	(54)
	0.9 - 4.6	adrenal hyperplasia	(8,10)
	0 - 1.2	infants and adult male	(10)
21-OH-pregnenolone	0.028	newborn infant	(21)
	0.120	puerperal female	(21)
	0.063	female	(21)
	0.025/1.	pregnant female (ACTH)	(90)
	0.070/1.	normal subjects	(90)
pregnenediol	0.50	males	(112)
	0.08 - 0.3	females	(112)
	0 - 4.1	adrenal tumor	(112)
	35/1.	adrenal tumor	(53)
	48/1.	adrenal tumor	(97)
	1.2 - 2.5	adrenal hyperplasia	(8,10)
	0.1/1.	adrenal hyperplasia	(82)
16α-OH-pregnenediol	0.008/1.	males	(35)
	6/1.	adrenal tumor	(55)
	≃2/1.	adrenal tumor	(39)
pregnenetriol	0.28/1.	males	(34)
	0.1 - 1.1	males	(112)
	0.19 - 0.48	females	(85)
	0.08 - 0.21	females	(27)
	0.2	females	(74)
	Tr - 0.36	females	(112)
	<0.2	females	(104)
	0.2 - 0.8	newborn infants and adults	(10)
	1.3/1.	adrenal tumor	(56)
	13	adrenal tumor	(94)
	3.7 - 53	adrenal tumor	(112)
	2.1	adrenal tumor	(87)
	3.3	adrenal tumor	(38)
	0.4 - 8.2	adrenal hyperplasia	(8,10)
	0.7 - 66	adrenal tumors and hyperplasia	(88)
	0.24 - 0.85	Stein-Leventhal syndrome	(27)
	0.2 - 1.0	Stein-Leventhal syndrome	(104)
	0.023 - 0.236	hypertensive males	(85)
20β-pregnenetriol	2.0	adrenal tumor	(38)

(continued)

Steroid	Amount	Subject	Reference
pregnenetetrol	0 - 5	adrenal hyperplasia	(10, 9)
	0.2	newborn infants	(10, 9)
DHA	0.2 - 4.5	males	(36)
	0.9 - 3.3	males	(112)
	0.3 - 0.4	males	(43)
	0.023 - 0.153	males	(58)
	2.1	male	(10)
	1.5	male	(5)
	3.4 ± 1.4	males	(68)
	0 - 2.0	females	(36)
	0 - 0.9	females	(112)
	0.63 - 3.8	females	(95)
	0.2 - 0.4	females	(43)
	0.041 - 0.104	females	(58)
	0.1 - 3.7	females	(60)
	0.3 - 4.0	females	(61)
	1.5	females	(5)
	1.2 ± 0.9	females	(68)
	0.065 - 0.108	pregnant women	(58)
	0.490	puerperal women	(5)
	0 - 0.008	newb orn infants	(21, 5)
	0.004	premature infants	(5)
	1.4 - 4.2	hirsute women	(43)
	0.4 ± 0.36	hirsute women	(13)
	0.4 - 0.8	polycystic ovaries	(112)
	1.4 - 204	adrenal tumors and hyperplasia	(88)
	< 0.01 - 0.2	polycystic ovaries	(74)
	2.1 ± 1.3	polycystic ovaries	(68)
	0.2 - 7.2	hirsute women	(71)
	0.4 - 8.3	hirsute women	(61)
	1.02 - 9.58	hirsute women	(95)
	0.1 - 3.7	hirsute women	(60)
	4 - 2.3	adrenal tumors	(112)
	351	adrenal tumor s	(54)
	110	adrenal tumors	(94)
	14.9	adrenal tumors	(38)
	702	adrenal tumors	(97)
	2.3 - 14	Cushing's disease	(59)
	1.4 - 4.5	adrenal hyperplasia	(8, 10)
androstenediol	0.2/1.	males	(34)
	0.5	males	(112)
	0 - 4.1	adrenal tumor	(112)
	0.18 - 24.5/1.	adrenal tumor	(97)
	7.7/1.	adrenal tumor	(53)
7α-OH-DHA	present	adrenal tumor	(87)

Steroid	Amount	Subject	Reference
7-keto-DHA	present	females	(62)
	present	"many normals"	(37)
	3.1	adrenal tumor	(37, 38)
	12/1.	adrenal tumor	(41)
16α-OH-DHA	0.05 - 0.15/1.	males	(34, 33)
	present	newborn infants	(92)
	0.2 - 0.3	newborn infants	(10)
	3.1	adrenal tumor	(87)
	0.3 - 3.5	adrenal hyperplasia	(8, 10)
7α, 16α-diOH-DHA	present	adrenal tumor	(87)
16α-OH-7-keto-DHA	present	adrenal tumor	(87)
	? present	adrenal hyperplasia	(10)
16-ketoandrostenediol (+16α-OH-DHA)	0.1 - 0.9	newborn infants	(93)
	0.3 - 1.4	adrenal hyperplasia	(93)
androstenetriol	0.1/1.	normal and pregnant subjects	(78)
	0.044/1.	male	(34)
	400/1.	adrenal tumor	(52)
16β-androstenetriol	0.036/1.	male	(34)

of production rates and excretory rates of appropriate compounds would yield quantitative information about intermediary metabolism. Elegant methods devised recently for the estimation of production rates of different steroids based upon urinary analyses after simultaneous administration of two differently-labeled compounds have extended the usefulness of urinary investigations(73). Preliminary data do not completely justify the assumptions of the mathematical model upon which the calculations of the rates were based. These data suggest that up to 50 mg./24 hours of DHA is produced in males and 25 mg./24 hours in females (107). In lieu of other direct methods of establishing production rates (this proposal was initiated in 1962), an indirect approach was conceived that was partially suggested by the findings of Shubert and Hobe and Stárka, et al. (102, 103). After the parenteral administration of DHA to normal or diseased subjects, investigators in both groups determined the amounts of the C-7 oxygenated metabolites of DHA. Shubert also determined 16α-OH-DHA and found ten times larger quantities than those he found for 7-keto-DHA. Stárka noted a direct relationship in the urinary values of DHA and 7α-OH-DHA although he did not measure the 16α-hydroxylated metabolite. DHA, unique among urinary 17-KS because it is also a precursor for other urinary 17-KS (androsterone and etiocholanolone), offers the possibility of being the key compound to further studies of androgen metabolism. Thus, a comparison of the urinary concentrations of DHA and of one of its metabolites (through reduction, hydroxylation, dehydrogenation or conjugation) could yield valuable information about the contribution of DHA to androgen metabolism if the transformations involved are neither

rate-limiting nor characteristic of a specific biochemical abnormality. The 16α-hydroxylated metabolite, 16α-OH-DHA, seemed to be an appropriate compound for that comparison on the basis of the limited excretory data available [(See Table 3)(102)]. The 16α-hydroxylase enzyme system is known to exist in the following human organs: liver (fetal and adult), adrenal gland (fetal and hyperplastic glands from adults) and ovary (23, 24, 28, 89).

If a constant ratio of urinary DHA/16α-OH-DHA exists in similar groups of subjects, a comparison of the output of urinary 16α-OH-DHA by normal and abnormal subjects will allow the estimation of DHA production differences in the compared groups by the use of the said ratio. The contribution to the urinary 17-KS by metabolic sequences other than the one characterized by the 3β-OH-Δ^5-steroids could be approximated by comparing the rates of production of DHA and of excretion of other 17-KS in the urine. In this way, the physiopathological events occurring in hirsutism, defeminization and virilism of females could be correleted. The objectives of this study were modified substantially in its early phases by the concurrent findings already mentioned. These will be discussed in another section. Emphasis centered upon the excretion rates of DHA and 16α-OH-DHA and their mutual relationship in efforts to fill a gap in our knowledge about this group of steroids.

II. MATERIALS AND METHODS:

A. CHEMICALS.

The reagents were analytical reagent grade with either the Baker and Adamson or Matheson, Coleman and Bell label unless specified differently. Any purification procedures that were introduced are described. The chloroform and ethyl acetate solvents were recovered after use by distillation in vacuo and reused without further purification. Aqueous solutions were prepared with water deionized with a Barnstead Deionizer, Model BD-1, using a standard cartridge.

Sulfuric Acid - Concentrated, 8 M and 5 M solutions.

Acetic Acid - Glacial, 50 percent (w/v) solutions.

Sodium Bicarbonate - Saturated solution.

Sodium Hydroxide - 0.1 M, 0.5 M and 2.5 M solutions.

Sodium Sulfate - Anhydrous granular salt.

Girard Reagent T - (Carboxymethyl) trimethylammonium chloride, hydrazide. (Only one grade was available.)

2-furaldehyde - Doubly distilled in vacuo. The mid-distillate was saved and stored at -14° C. A solution composed of 0.56 percent (v/v) in 50 percent acetic acid (w/v) was stable at 4° C.

Tetramethylammonium Hydroxide - 1 percent (w/v) in 95 percent ethanol.

Blue Tetrazolium [3:3' - dianisolebis - 4:4' -(3:5 - diphenyl) tetrazolium chloride] - 0.07 percent (w/v) in 95 per-

cent ethanol. Lot BA3968, Mann Research Laboratories, Inc.

Methyl Alcohol - Absolute and 90 percent (v/v) solutions.

Ethyl Alcohol - (Commercial Solvents Corp.) Absolute and 95 percent (v/v) solutions. The 95 percent solution for the Oertel-Eik-Nes reaction was prepared daily after freshly distilling absolute ethyl alcohol in vacuo with an excess of silver oxide (Ag_2O - Mallinkrodt).

Dichloromethane

Benzene

Chloroform

Silica gel - 100-200 mesh (Will Corp.) activated periodically by placing at 120° C for 2 hours. It was stored in a stoppered flask.

Steroid Standards

Dehydroepiandrosterone, Schering Co., Berlin. M.P. 151.5 - 152.5° C.

Dehydroepiandrosterone-sulfate (sodium salt), Mann Research Lab., Lot B3696.

3β, 16α-dihydroxyandrost-5-en-17one, prepared by Dr. A. Colás from synthetic 3β, 16α, 17α-trihydroxypregn-5-en-20-one by periodate oxidation (26). M.P. 183-188° C.

3β, 16α, 17α-trihydroxypregn-5-en-20-one (diacetate). A sample prepared by Dr. Colás from 16-dehydropreg-

nenolone by treatment with $KMnO_4$ in cold acidified acetone followed by room temperature acetylation with acetic anhydride and pyridine (26). M. P. 208-211° C.

3β, 16α-dihydroxy-5-en-17-one (diacetate). A sample prepared by Dr. Colás from 3β, 16α, 17α-trihydroxypregn-5-en-20-one (diacetate) through oxime formation and $POCl_3$ treatment (42). M. P. 166-170°

Androst-5-ene-3β, 16α, 17α-triol. A gift from Dr. A. Ercoli. M. P. 261-264° C.

3β, 7α-dihydroxyandrost-5-en-17-one. A gift from Dr. D. Fukushima. (M. P. not given.)

B. EXTRACTION AND SOLVOLYSIS.

Urine specimens were collected as 24-hour samples and were kept refrigerated or iced during collection. Specimens were processed immediately or frozen (-14° C.) to be analyzed when the various steps in the procedure could be accomplished without interruption. The solvolysis procedure designed by Burstein and Lieberman (15) to hydrolyze ketosteroid hydrogen sulfates was used in this study. After titration of an aliquot of urine, enough 8 M sulfuric acid was added to one-fifth of a 24-hour sample to bring it to pH 2. The acidified urine was extracted once with an equal volume of ethyl acetate. The extract was placed at 38° C for a minimum of 15 hours. After hydrolysis, the extract was washed with one-fifth volumes of saturated sodium bicarbonate and of water. The extract was dried

with 5 percent sodium sulfate, filtered through Whatman No. 1 paper into a flask and evaporated to dryness with reduced pressure.

C. PURIFICATION.

Results of experiments that confirmed the need for additional purification are given later. A chemical purification by separation of ketonic and nonketonic compounds results from the addition of Girard's reagent T ([carboxymethyl]trimethyl ammonium chloride, hydrazide) (45) with acid-alcohol as solvent. For Girard separation, 0.1 ml. of a mixture of equal parts of glacial acetic acid and 90 percent methanol was added for each mg. of dry residue in the extract. After adding 0.01 g. of the hydrazide per mg. of dry residue, the reaction mixture was left overnight at room temperature (21 -24° C.). In the presence of 3 volumes of 2.5 M sodium hydroxide and 10 volumes of water, the hydrazones were water soluble and were not extracted during 6 rapid chloroform (10 volumes each) extractions (cold reagents are used to impede oxidation during the extraction of the nonketonic compounds). Lowering the pH of the aqueous phase to 2 (0.24 ml. of 5 M H_2SO_4 for each mg. dry residue) favors the oxidation of the hydrazones. Under these conditions, the ketones were extracted during 6 chloroform (10 volumes each) extractions in 2 hours. The combined ketonic extracts were washed with 0.1 M NaOH (1/5 volume) and each of the combined extracts was washed with 1/5 volume of water. Each was dried with 5 percent sodium sulfate, filtered and evaporated in vacuo to dryness for column chromatography.

D. COLUMN CHROMATOGRAPHY.

A method for the chromatographic separation of polar adrenal steroid hormones devised by Katzenellenbogen et al (66), was adopted for this study. They reported that a system consisting of ethanol adsorbed on silica gel as the stationary phase and methylene chloride containing 1 to 5 percent ethanol as the mobile phase was satisfactory for the resolution of most of the steroids they sought in urinary extracts. The stationary phase was prepared by continuously mixing absolute ethanol added drop by drop to 10 g. of silica gel until it was just saturated (4.2 ml.). Mobile phase was added to make a slurry which was poured into a 1x50 cm. column prepared by placing a filter paper disc over a fritted glass disc-stopcock terminal and prefilled with methylene chloride. A firm gel resulted when the glass column was tapped firmly as the gel settled in the solvent which was allowed to escape simultaneously. The final silica gel column was 1x18 cm. Another filter paper disc was placed on top of the silica gel which was washed with 4 volumes of methylene chloride before the steroid sample was applied in the smallest-possible volume of solvent (about 2 - 3 ml.). This solution was allowed to seep into the adsorbent before additional mobile phase was added. The final mobile phase consisted of 320 ml. of ethanol in methylene chloride in the following amounts: 20 ml. of 0.5 percent ethanol; 100 ml. of 1 percent ethanol; 60 ml. of 2 percent ethanol; 60 ml. of 4 percent ethanol; 40 ml. of 8 percent ethanol; 20 ml. of 16 percent ethanol, and 20 ml. of absolute ethanol. Fractions of 18.5 ml. were collected with the aid of an automatic fraction collec-

tor. Satisfactory resolution occurred if the solvent was allowed to flow at the rate of approximately 100 ml. per hour. Two milliliter aliquots of each fraction were dried in tubes for colorimetry using a stream of nitrogen and a 50° C. water bath.

E. COLORIMETRY.

Munson, et al., modified the Pettenkofer colorimetric procedure to the quantitation of 3β-OH-Δ^5-steroids (83). Fotherby (36) adapted the reaction to a micro-technique which we used in preliminary tests on reliability. Further volume reduction has been used in most of the experiments. To facilitate the accurate addition of small amounts of reagents 2 ml. of a freshly prepared mixture of glacial acetic acid, 2-furaldehyde-acetic acid solution and 8 M H_2SO_4 (1:4:15) were added to each glass-stoppered reaction tube. After mixing to dissolve the residue, the samples, which included duplicate DHA standards (10 and 20 µg.) and a blank, were placed in a 67° C. constant temperature water bath for 12 minutes for color development. They were next placed in an ice bath to inhibit further reaction and read immediately at 635, 660 and 685 mµ in a Zeiss Model PMQ II spectrophotometer. Maximum absorbance for 3β-hydroxy-Δ^5-steroids in the modified Pettenkofer reaction is at 660 mµ. The absorbance measured at 635 and 685 mµ, wave lengths that are adjacent and equidistant from the maximum, are utilized in the application of Allen's Correction (2):

$$A_{corrected} = A_{max.} \quad \frac{(A_{max.} - 25\,m\mu + A_{max.} + 25\,m\mu)}{2}$$

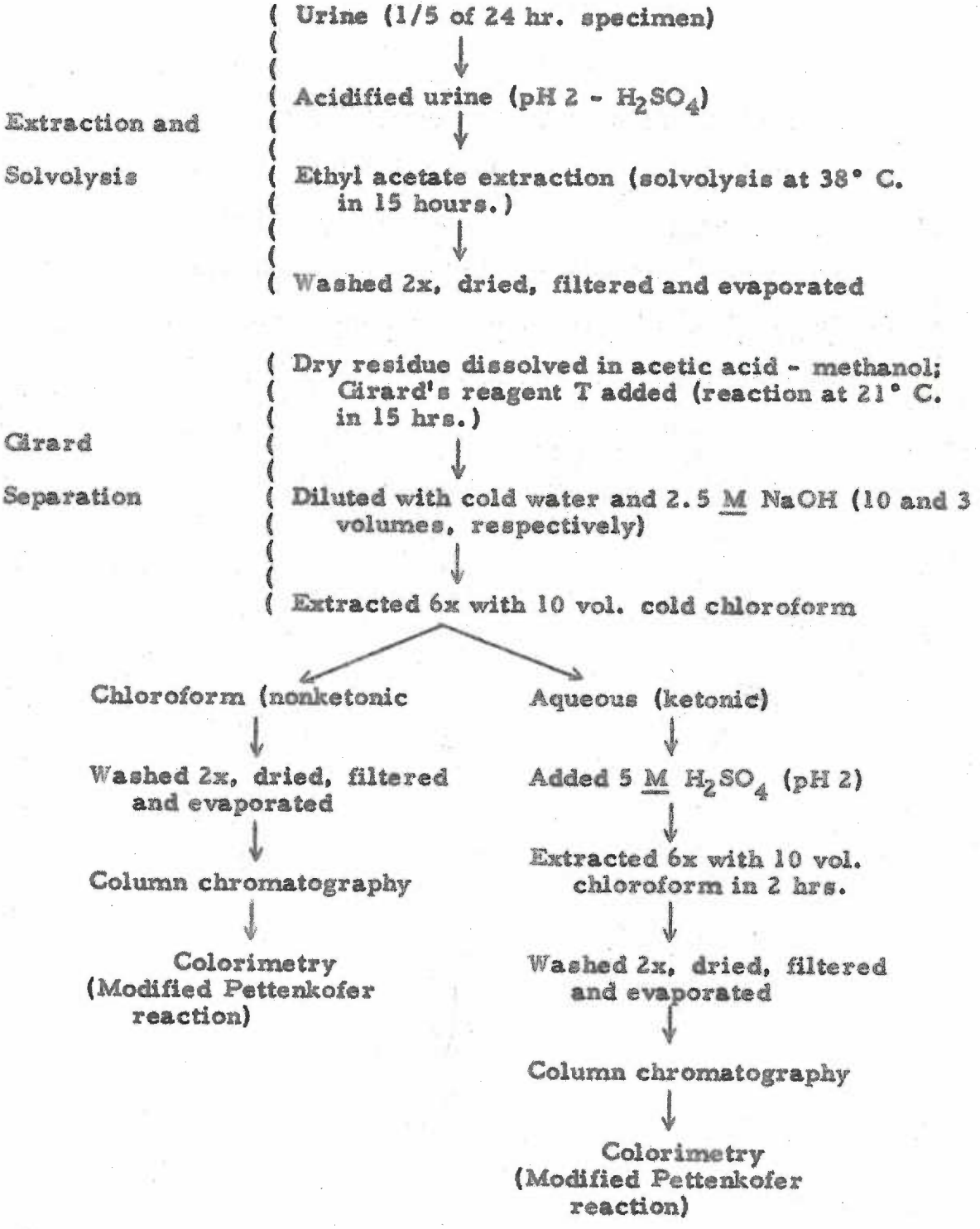
Results are expressed as mg. excreted per 24 hours.

A method for quantitation of 3α-OH-Δ^5-steroids presented by Oertel and Eik-Nes (86) is based upon the chromogen formed in the presence of ethanol and sulfuric acid. The reagent is prepared by adding 1 volume of 95 percent ethyl alcohol (freshly distilled over Ag_2O) and 2 volumes of concentrated sulfuric acid. The dry residue is dissolved in 2.5 ml. of the reagent and allowed to react at room temperature. After 2 to 15 minutes, the yellow solution is read spectrophotometrically against a blank at 380, 405 and 430 mμ. The Allen Correction (2) was applied to the raw data.

F. OTHER IDENTIFICATION PROCEDURES.

Paper partition chromatographic systems devised by Bush (16) for steroid separations were used for identification of some fractions from urine extracts. Glass tanks were lined with paper that increased saturation of the atmosphere by absorbing mobile phase which had been poured into the tank. Stationary phase was placed in a dish in the bottom of the tank. After 'spotting' the steroids on the paper, the paper was allowed to equilibrate overnight before the mobile phase was poured into the trough through holes in the tank lids to initiate development which took about 8 hours. The solvent system consisted of benzene, methanol and water (10:7:3 by volume). The blue-tetrazolium reaction as modified by Fotherby (34) was applied to some fractions of urine extracts to aid identification. To the dry residues, 0.25 ml. of 1 percent (w/v) tetramethylammonium hydroxide in 95 percent ethanol and 0.35 ml. of 0.07 percent (w/v) blue tetrazolium in 95 percent ethanol are added for incubation for 1 hour in the dark at 25° C. Thereafter, 4 ml. of 1 percent (v/v) acetic acid in 50 percent ethanol was added

FIGURE NO. 7. Summary of procedures that compose a method for urinary 3$^{\alpha}$-OH-Δ^{5}-steroids.


Urine (1/5 of 24 hr. specimen)
Acidified urine (pH 2 - H_2SO_4)
Extraction and Solvolysis
Ethyl acetate extraction (solvolysis at 38° C. in 15 hours.)
Washed 2x, dried, filtered and evaporated
Girard Separation
Dry residue dissolved in acetic acid - methanol; Girard's reagent T added (reaction at 21° C. in 15 hrs.)
Diluted with cold water and 2.5 M NaOH (10 and 3 volumes, respectively)
Extracted 6x with 10 vol. cold chloroform
Chloroform (nonketonic
Washed 2x, dried, filtered and evaporated
Column chromatography
Colorimetry (Modified Pettenkofer reaction)
Aqueous (ketonic)
Added 5 M H_2SO_4 (pH 2)
Extracted 6x with 10 vol. chloroform in 2 hrs.
Washed 2x, dried, filtered and evaporated
Column chromatography
Colorimetry (Modified Pettenkofer reaction)

and the absorbance measured at 490, 520 and 550 mμ. The Allen Correction was applied. 16α-OH-DHA (diacetate) was used for the comparative standard.

III. RESULTS

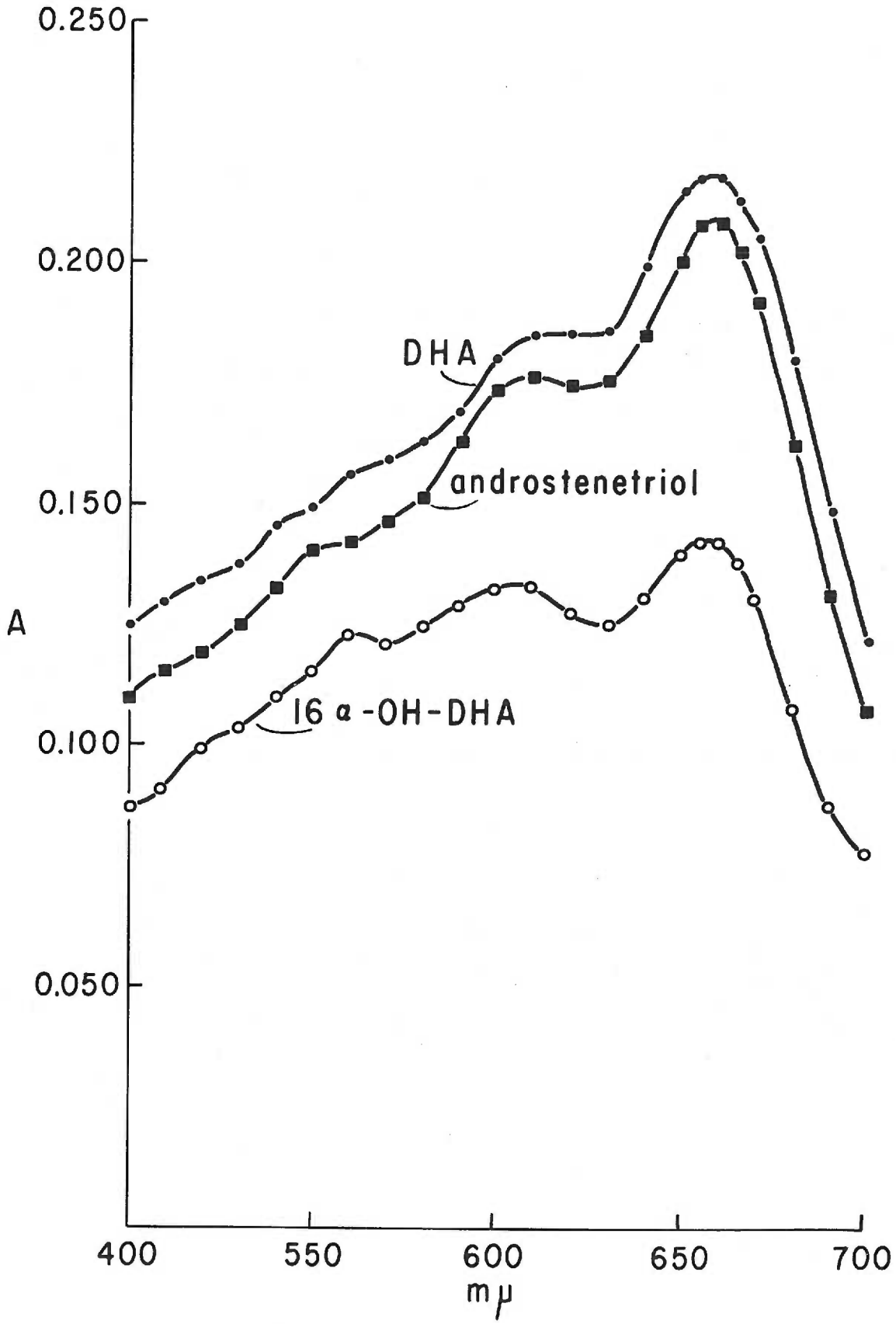
A. RELIABILITY.

The procedure finally adopted for the quantitation of 3β-hydroxy-Δ^5-steroids in urine represents a new combination of physical-chemical operations which had found separate application previously in steroid analysis. Experiments were therefore conducted to establish the reliability of each operation and of the entire method.

1. Quantitation: The modified Pettenkofer reaction between 3β-hydroxy-Δ^5-steroids and furfural in the presence of concentrated sulfuric acid was studied for specificity, precision, sensitivity and accuracy. The spectra of the following 3β-OH-Δ^5-steroids that might be anticipated in urine demonstrated maximum absorbance at 660 mμ: DHA, 7α-OH-DHA, 16α-OH-DHA, androstenetriol and 16α, 17α-diOH-pregnenolone (diacetate). Typical spectra are presented in Fig. 8.

   The chromogenic response to increasing amounts of DHA did not obey Lambert-Beer's Law and deviated greatly at the higher concentrations tested. Reasonable linearity obtained with up to 10 μg. (2 ml. reaction mixture) but at 20 μg. the extinction coefficient had

FIGURE NO. 8. Spectral analysis of some 3β-OH-Δ^5-steroids (5 μg./ml.) in the modified Pettenkofer reaction.


0.250
0.200
0.150
0.100
0.050
A
DHA
androstenetriol
16 α -OH-DHA
400
550
600
650
700
mμ

decreased to 85 percent of that at the lower concentration, and at 40 μg. was only 64 percent. The introduction of Allen's Correction decreased the linearity slightly for pure compounds but improved linearity of the chromogenic response in urinary extracts. These data are plotted in Fig. 9.

Using the 4 ml. reaction mixture, the precision achieved measuring 30 μg. aliquots of DHA in each of 12 samples was ± 0.24 μg. (± 0.06 μg./ml.) when precision is defined as equivalent to one standard deviation from the arithmetic mean.*

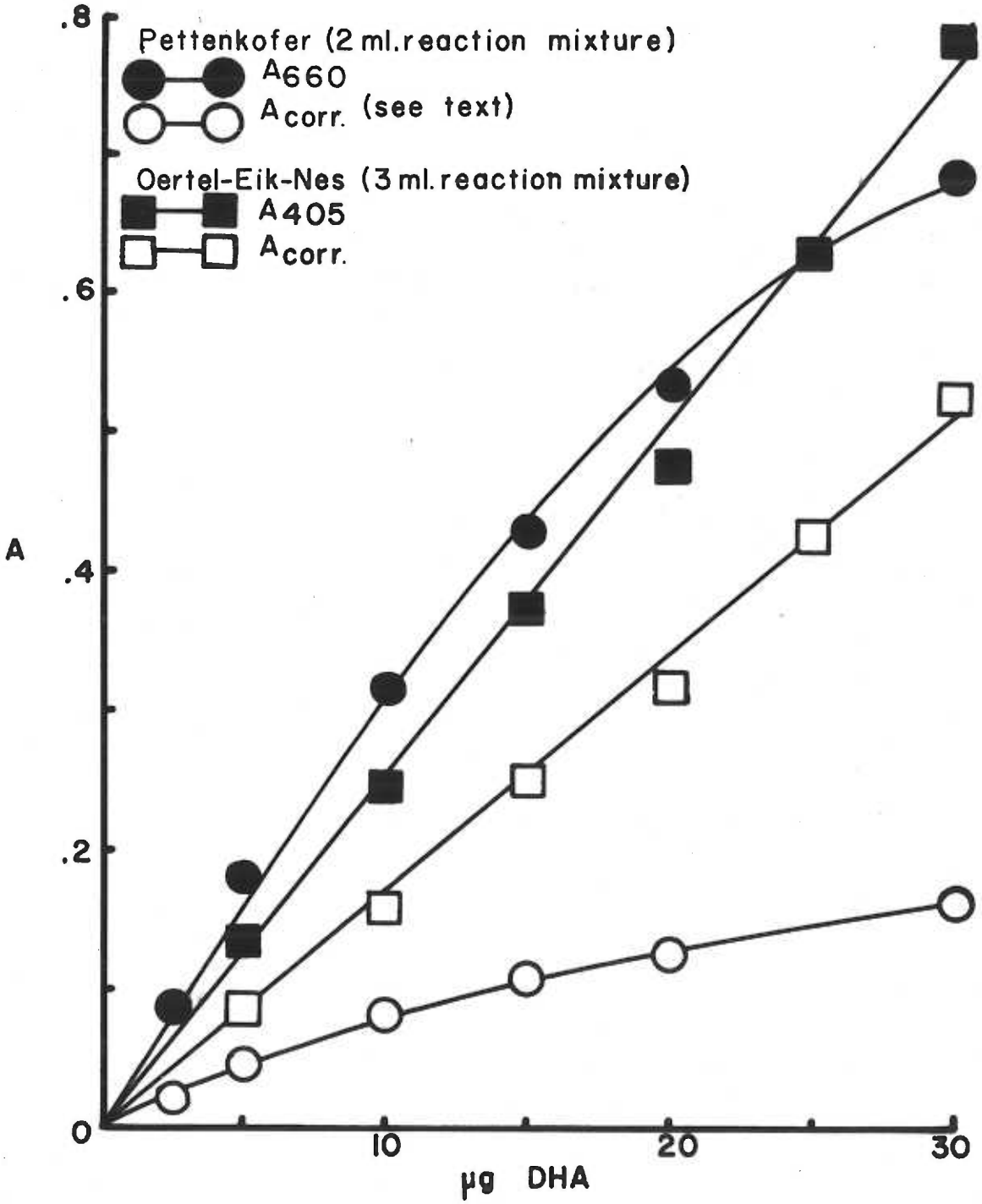
The sensitivity of the chromogen was 0.48 μg. (0.12 μg./ml.) when 30 μg. of DHA were quantitated. Sensitivity is defined as twice the standard deviation.*

Similar experiments using the Oertel-Eik-Nes reaction demonstrated that the precision for 10 μg. of DHA was ± 0.18 μg. (± 0.06 μg./ml.) based on 20 samples and sensitivity was 0.36 μg. (0.12 μg./ml.). The chromogenic response at different concentrations was nearly linear over the range 5 - 30 μg. (3 ml. reaction mixture) and was linear after application of Allen's Correction. Significant deviations in linearity existed at 40 μg. concentrations.

The spectral analysis of the Oertel-Eik-Nes chromogens with the steroid standards disclosed inconsist-

*See discussion by Brown, J. G., Bulbrook, R. D., and Greenwood, F. C., J. Endocrinol., 1957, 16, 41-48.

FIGURE NO. 9. Plot of the true and of the corrected absorbance at the wave lengths of maximum absorbance against different concentrations of DHA in the modified Pettenkofer and the Oertel-Eik-Nes reactions.


.8
.6
.4
.2
0
A
Pettenkofer (2 ml. reaction mixture)
A660
Acorr. (see text)
Oertel-Eik-Nes (3 ml. reaction mixture)
A405
Acorr.
10
20
30
µg DHA

encies of the wave length of maximum absorbance. The maximum for DHA was 405 mμ; for 16α-OH-DHA, 380 mμ; for 7α-OH-DHA, 580 mμ; and for androstenetriol, 405 mμ. Rapid changes in the absorption maxima occurred within a short time after the reaction was initiated. The absorption spectrum was especially complex and the absorption changes in time pronounced for 16α-OH-DHA. Representative data are given in Figs. 10 and 11.

2. Extraction and solvolysis: The extraction and solvolysis operations yielded 91 percent of DHA as the free steroid in 15 hours after the sodium sulfate conjugate was added to water, extracted, solvolyzed, collected, washed and quantitated. A duplicate sample yielded 78 percent of the added quantity after the additional introduction of silica gel column chromatography. (See Table No. 4.)

3. Column chromatography: Addition of DHA and 16α-OH-DHA directly onto a silica gel column followed by elution with methylene dichloride-ethanol solvents yielded 89 and 88 percent respectively of the original quantity. For experiments testing recoveries of 16α-OH-DHA, small quantities of that compound were used for comparative standards but otherwise, DHA was the standard. The resolution of DHA and 16α-OH-DHA by the column was satisfactory without significant overlap or tailing and is illustrated in Fig. 12.

FIGURE NO. 10. Spectral analysis of some 3β-OH-Δ^5-steroids (6 μg./ml.) in the Oertel-Eik-Nes reaction.

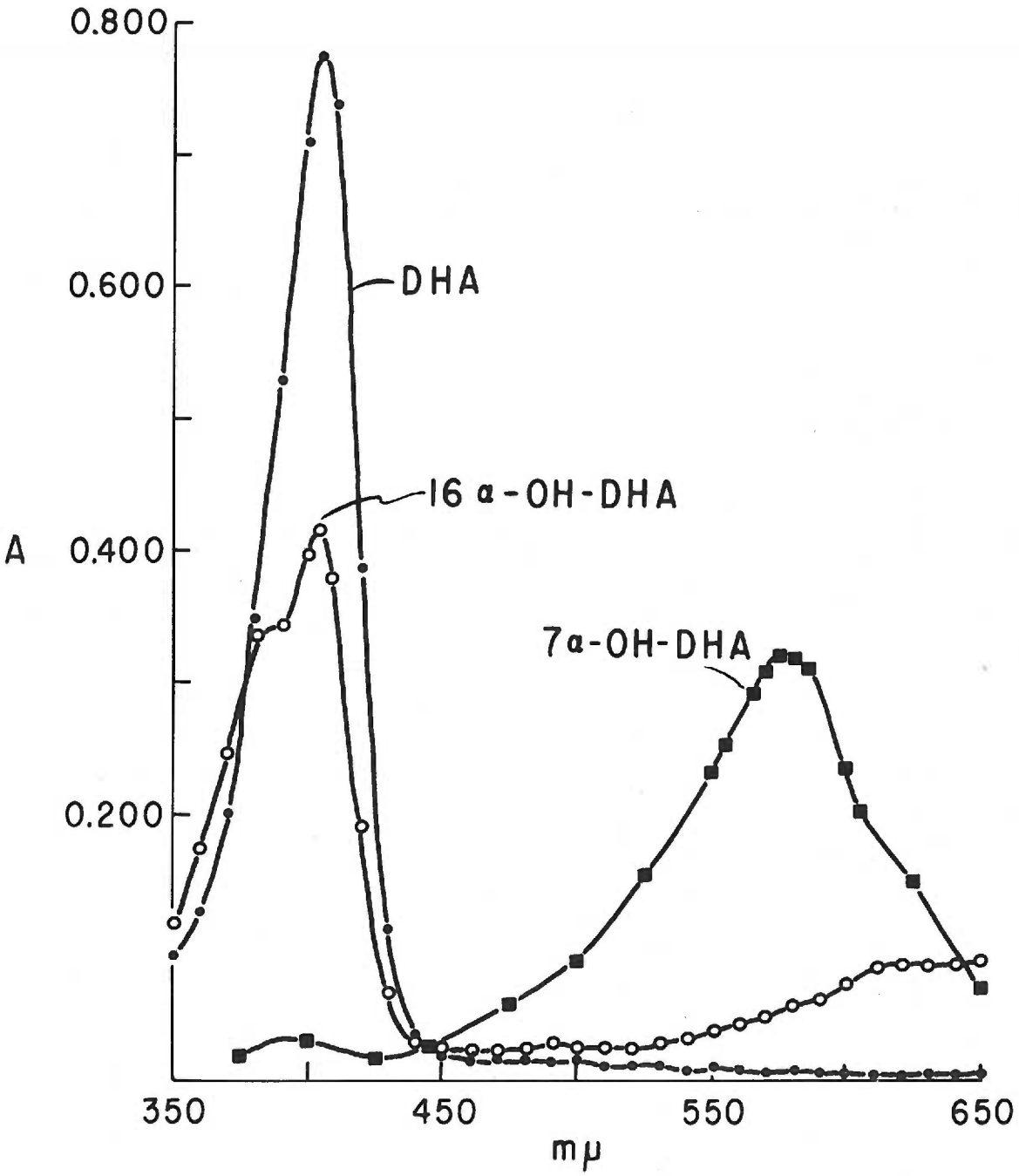

0.800
0.600
0.400
0.200
A
DHA
16 α-OH-DHA
7α-OH-DHA
350
450
550
650
mμ

FIGURE NO. 11. Absorbancy changes with time for DHA and 16α-OH-DHA in the Oertel-Eik-Nes reaction. The reaction time in the upper figure was 15 minutes; in the lower figure, 2 hours.

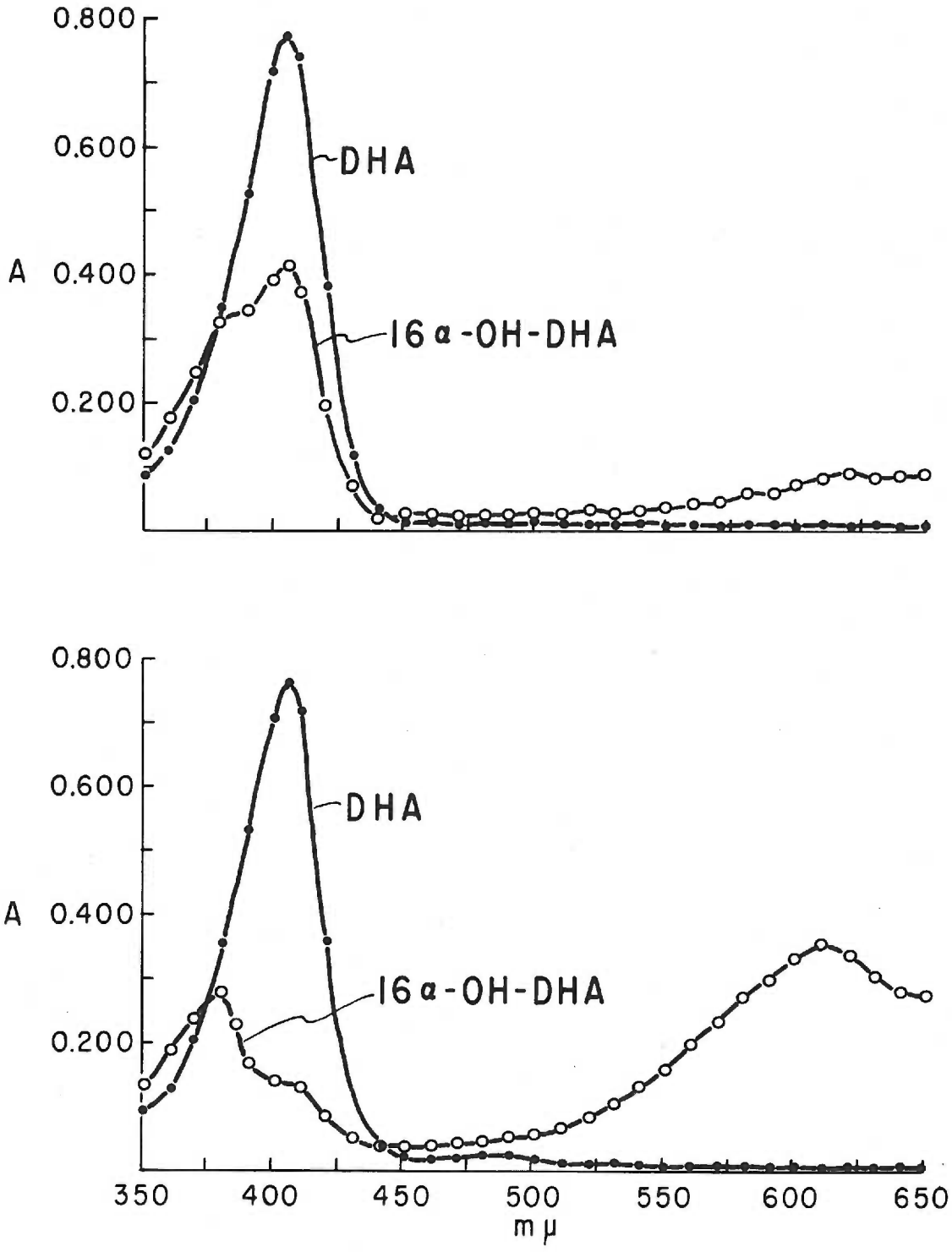
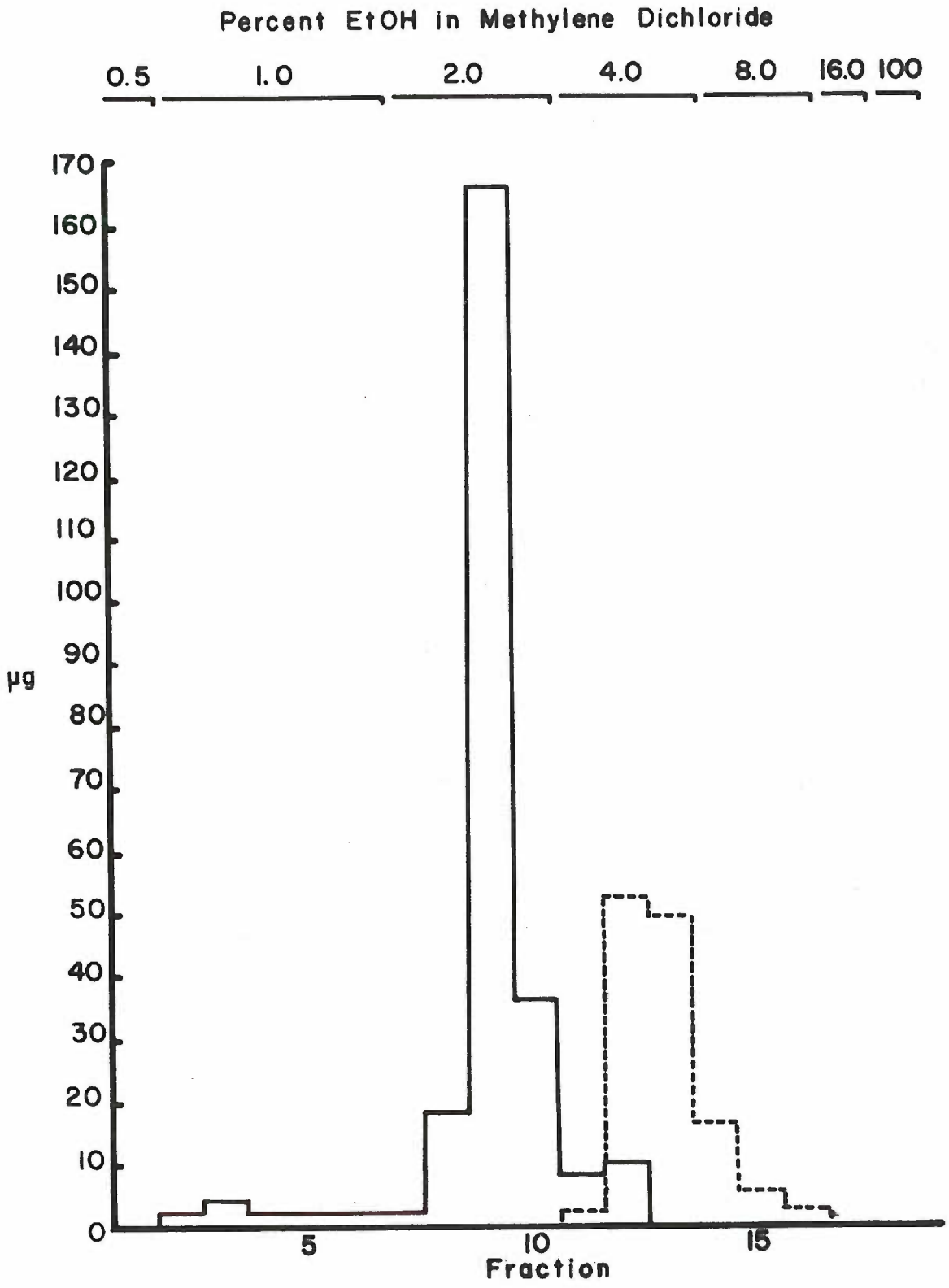

0.800
0.600
A 0.400
0.200
DHA
16α-OH-DHA
0.800
0.600
A 0.400
0.200
DHA
16α-OH-DHA
350
400
450
500
550
600
650
mμ

TABLE NO. 4. Determination of the accuracy of the method: percent recovery of 0.100 mg. DHA-SO_3-Na from water and urine (equivalent to 0.074 mg. free DHA).

	From water		From urine	
		Chromatography		Chromatography
Extraction and Solvolysis	91	78	-	-
Extraction, Solvolysis and Girard sep'n	68	73	69	68

FIGURE NO. 12. Column chromatographic resolution of pure steroids. Recovery of DHA at the 0.250 mg. level was 89 percent; of 16α-OH-DHA at the 0.138 mg. level, 88 percent. In the histogram the solid lines represent DHA and the interrupted lines represent 16α-OH-DHA.

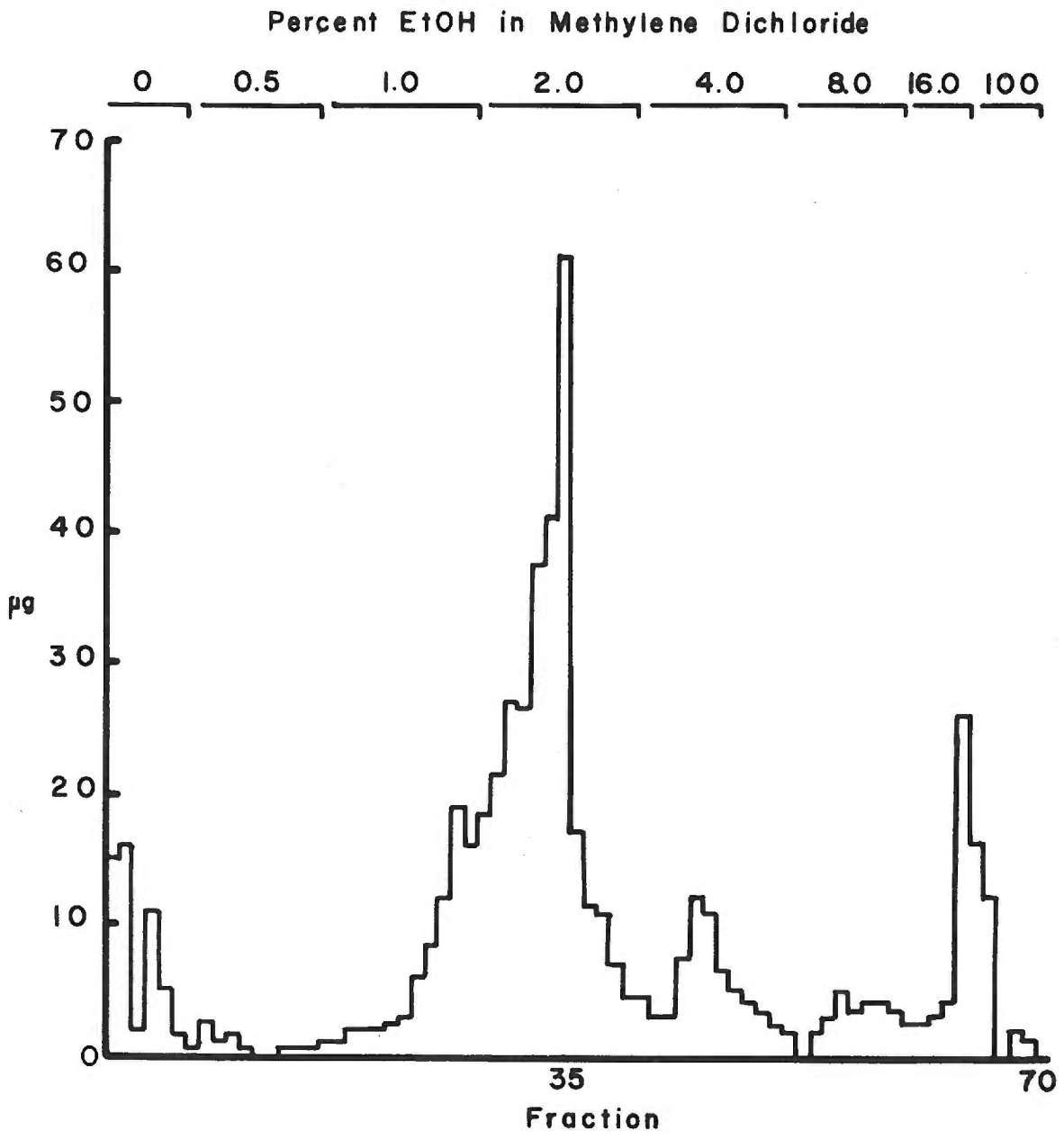

Percent EtOH in Methylene Dichloride
0.5
1.0
2.0
4.0
8.0
16.0
100
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
µg
5
10
15
Fraction

4. Girard separation: The application of the extraction and solvolysis, column chromatography and quantitation operations to urine indicated the need for additional procedural alterations. (See Fig. 13.) The presence of unidentified lipid-like compounds that were eluted initially and pigments with similar polarity as DHA added uncertainty to the procedure. The introduction of the Girard separation of ketonic and nonketonic compounds removed the abundant offending compounds. In addition, the accumulation of fewer but larger fractions did not affect the resolution. (See Fig. 14.) Girard separation conditions cause a decrease in the recovery of steroid sulfates added to water from 91 to 68 percent if no chromatography was introduced and from 78 to 73 percent if chromatography was employed. Recoveries from urinary extracts were 69 and 68 percent, respectively, for those operations. (See Table No. 4.)

The adequacy of the Girard separation was investigated by subjecting column Fraction No. 12 after this treatment to paper chromatography. The ketonic fraction contained Pettenkofer positive spots with R_f values of 0.63 and 0.94, the former identical with that of pure 16α-OH-DHA in this system. The nonketonic fraction had a single spot with the value 0.74 but none with the mobility of 16α-OH-DHA.

5. Confirmation of the combined procedures: In addition to

FIGURE NO. 13. Column chromatographic resolution of steroids in a crude urinary extract.


Percent EtOH in Methylene Dichloride
0
0.5
1.0
2.0
4.0
8.0
16.0
100
70
60
50
40
μg
30
20
10
0
35
70
Fraction

the recovery experiments with steroid conjugates (Table 4), the recovery of free DHA added to urine after extraction and solvolysis yielded 83 percent of 250 μg. and 92 percent of 125 μg. in separate experiments and 69 percent of 661 μg. and 66 percent of 184 μg. of added 16α-OH-DHA estimated simultaneously. The values for 16α-OH-DHA have not been corrected for the lower molar extinction coefficient of that compound in comparison to DHA nor have other correction factors been introduced. These data are graphed in Fig. 15.

The recovery experiments provided evidence for the distribution of pure compounds among the fractions collected as well as information about the accuracy of the method. The identification of components in specific fractions of some urinary extracts was further supported with paper chromatographic analysis and the blue tetrazolium reaction.

Fraction No. 12 of the ketonic material from subject No. 4 was submitted to paper chromatography, along with pure 16α-OH-DHA as a separated standard. The pure 16α-OH-DHA had an R_f of 0.62 compared to 0.63 for the urinary fraction. The R_fs were determined by cutting the paper transversely into strips from which the steroids were eluted individually with methanol and quantitated by the Pettenkofer reaction. About ninety

FIGURE NO. 14. Column chromatographic resolution of the ketonic fraction of a urinary extract after Girard purification.

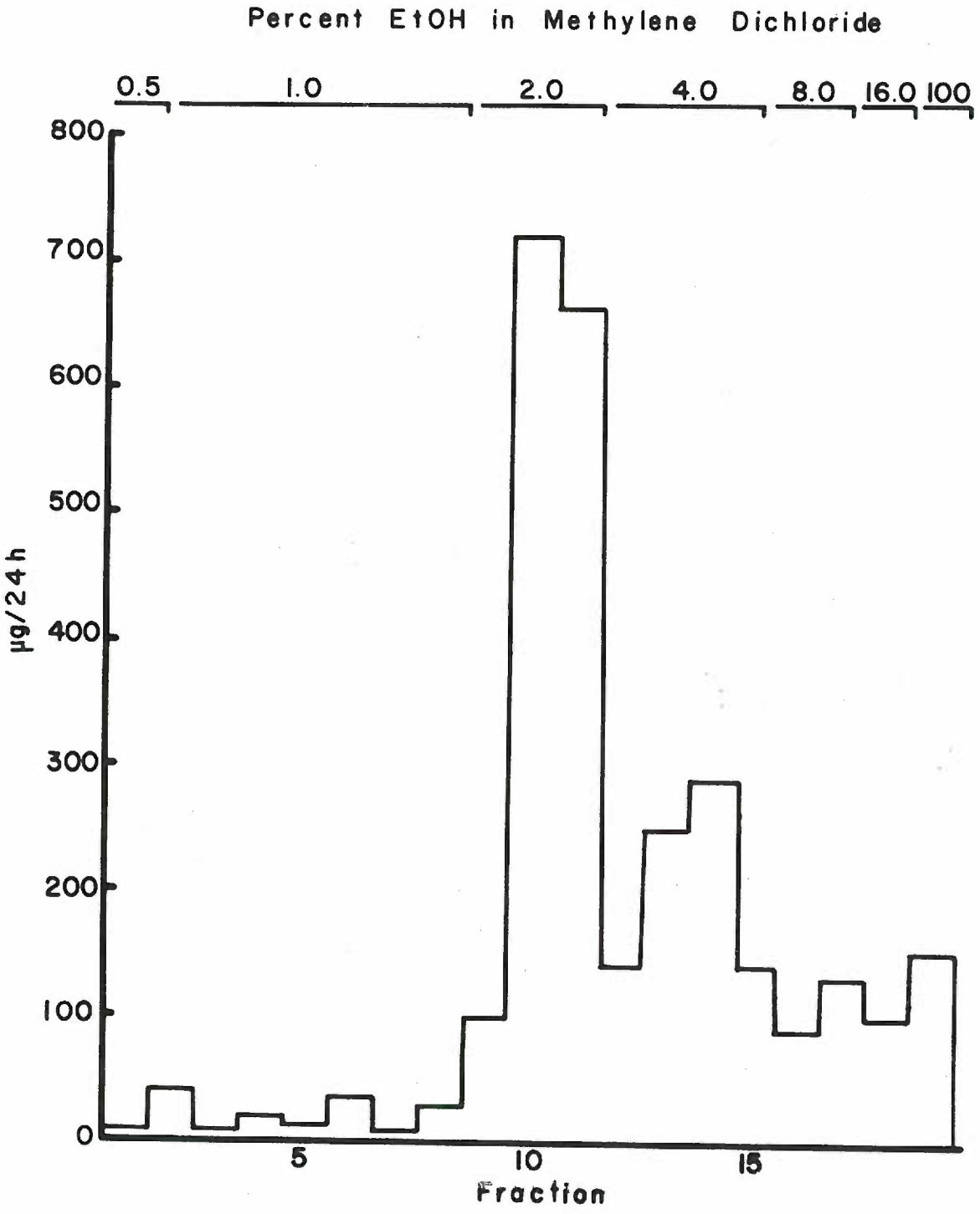
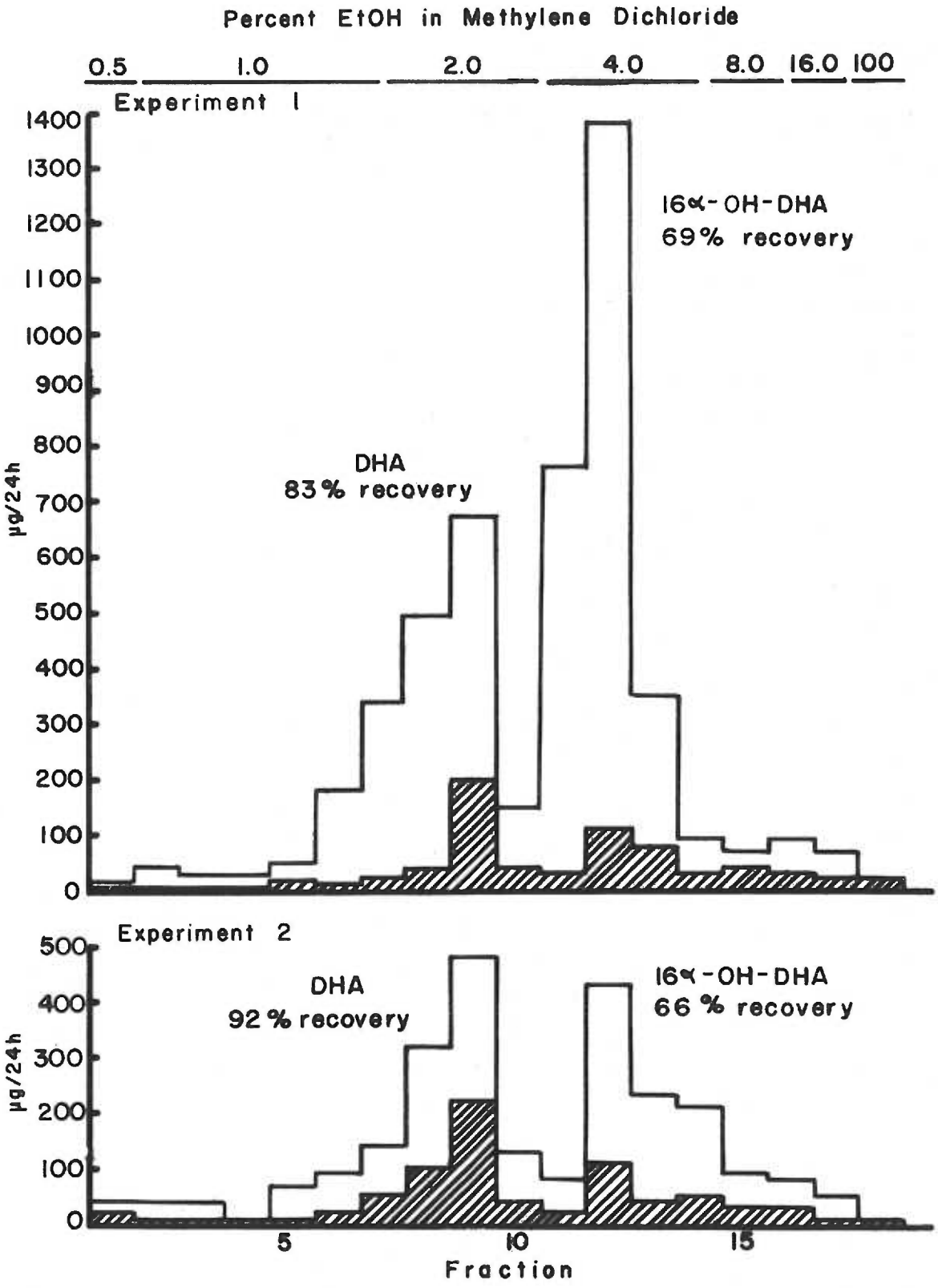

Percent EtOH in Methylene Dichloride
0.5
1.0
2.0
4.0
8.0
16.0
100
800
700
600
500
400
300
200
100
0
µg/24 h
5
10
15
Fraction

FIGURE NO. 15. Determination of the accuracy of the method: percent recovery of 0.250 and 0.125 mg. pure DHA and 0.661 and 0.184 mg. pure 16α-OH-DHA, respectively, from urinary extracts in two separate experiments. The cross-hatched areas in the histograms represent the values obtained before the addition of pure compounds.


Percent EtOH in Methylene Dichloride
0.5
1.0
2.0
4.0
8.0
16.0
100
Experiment 1
16α-OH-DHA
69% recovery
DHA
83% recovery
µg/24h
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Experiment 2
DHA
92% recovery
16α-OH-DHA
66% recovery
µg/24h
500
400
300
200
100
0
5
10
15
Fraction

percent of the Pettenkofer positive material in the ketonic fraction No. 12 appeared in the R_f 0.62 spot but about 10 percent migrated in a spot with an R_f of 0.94.* An aliquot of fraction No. 12 subjected to the blue tetrazolium reaction gave a positive color reaction denoting an α-ketolic component. In another experiment, Pettenkofer positive material from the 12th ketonic fraction from Subject 9 also migrated in the same chromatographic system with pure 16α-OH-DHA as determined by the blue tetrazolium reaction.

The nonketonic fraction No. 12 from Subject No. 4 was chromatographed on paper using pure androstenediol and pure androstenetriol as standards. The pure androstenediol R_f value determined in the same way as before was 0.73 and the R_f value of a single spot in Fraction 12 was 0.74.

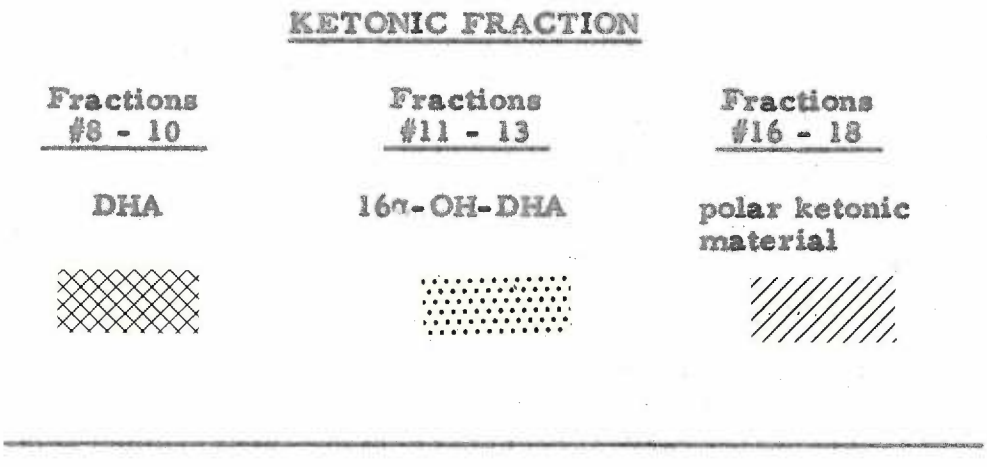
B. URINARY EXTRACTS.

The excretion patterns of 3β-OH-Δ^5-steroids were determined in some normal subjects for comparison with those in some patients. These data appear in Figs. No. 16 - 22 in chromatographic-pattern form and in Tables No. 5 - 11 in digital form.

1. Normal subjects: Except for one subject, the males (ages 19 - 35 years) excreted more DHA than 16α-OH-DHA. The mean urinary content of DHA (fractions 8 - 10)

* The fractions designated in this thesis as DHA and 16α-OH-DHA represent mainly those compounds but may also include other Pettenkofer positive material, with similar chromatographic mobilities.

FIGURE NO. 16. Urinary 3β-OH-Δ^5-steroids from normal males. Shaded areas on each plot correspond to accumulated fractions (see Table No. 5). Ordinates are in mg./24 h.



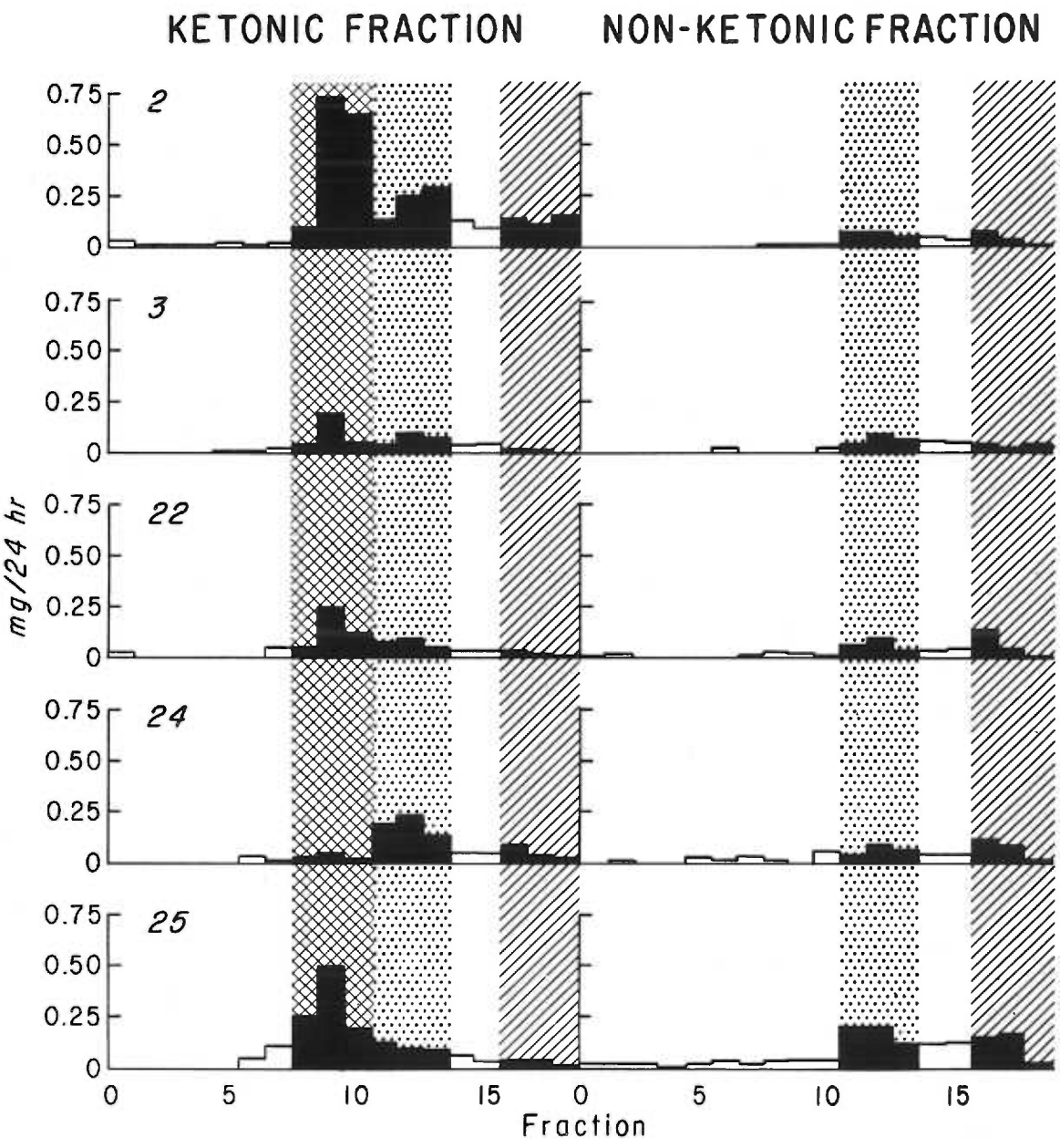

KETONIC FRACTION
NON-KETONIC FRACTION
2
3
22
24
25
0.75
0.50
0.25
0
mg/24 hr
0
5
10
15
Fraction

FIGURE NO. 17. Urinary 3β-OH-Δ^5-steroids from normal females. Shaded areas on each plot correspond to accumulated fractions (see Table No. 6). Ordinates are in mg. / 24 h. Refer to the legend of Figure No. 16 for the explanation about the different accumulated fractions.

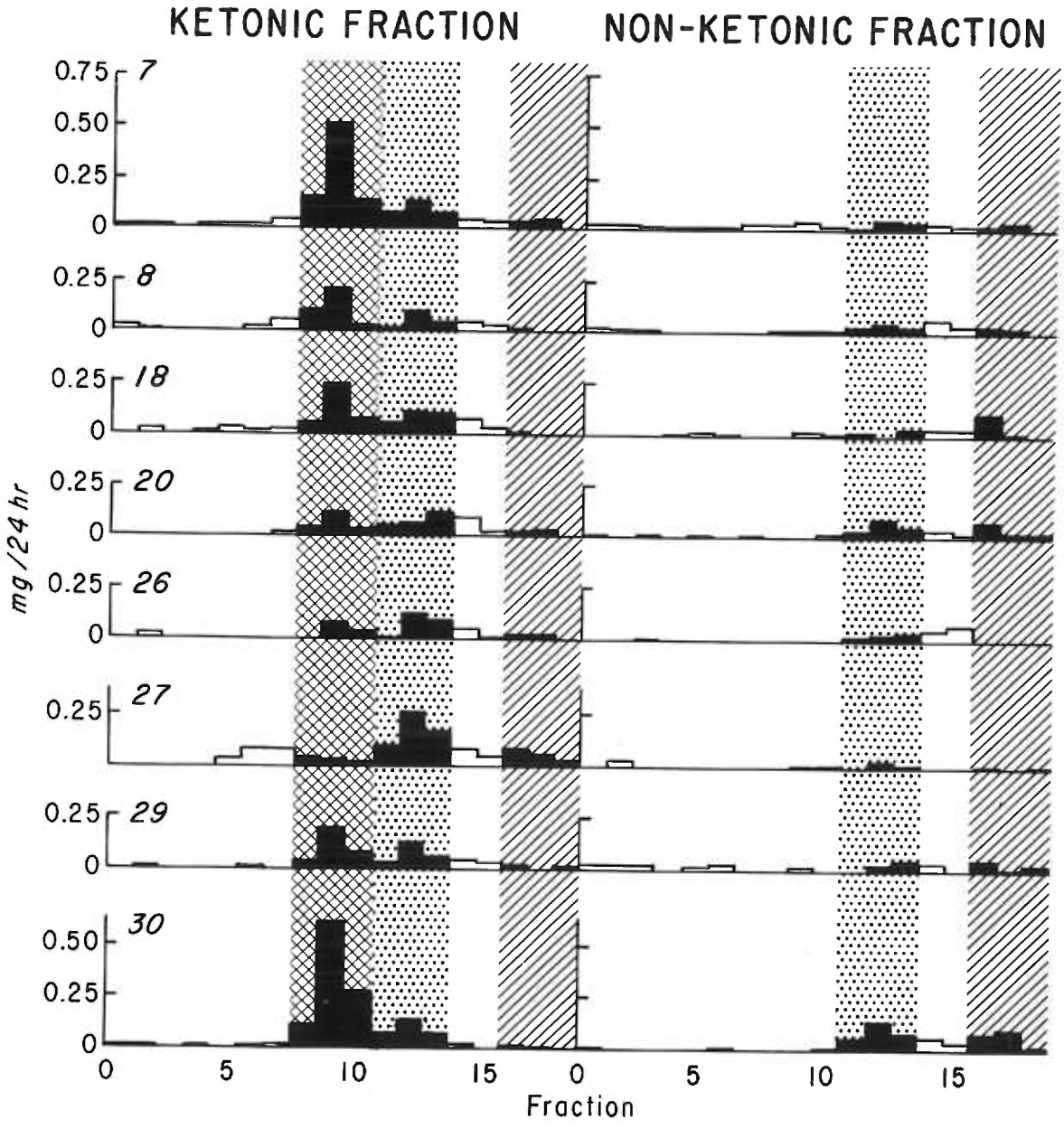

KETONIC FRACTION
NON-KETONIC FRACTION
mg/24 hr
0.75
0.50
0.25
0
7
0.25
0
8
0.25
0
18
0.25
0
20
0.25
0
26
0.25
27
0.25
0
29
0.50
0.25
0
30
0
5
10
15
0
5
10
15
Fraction

FIGURE NO. 18. Urinary 3β-OH-Δ^5-steroids from normal pregnancies (see Appendix for duration). Shaded areas on each plot correspond to accumulated fractions (see Table No. 7). Ordinates are in mg./24 h. Refer to the legend of Figure No. 16 for the explanation about the different accumulated fractions.

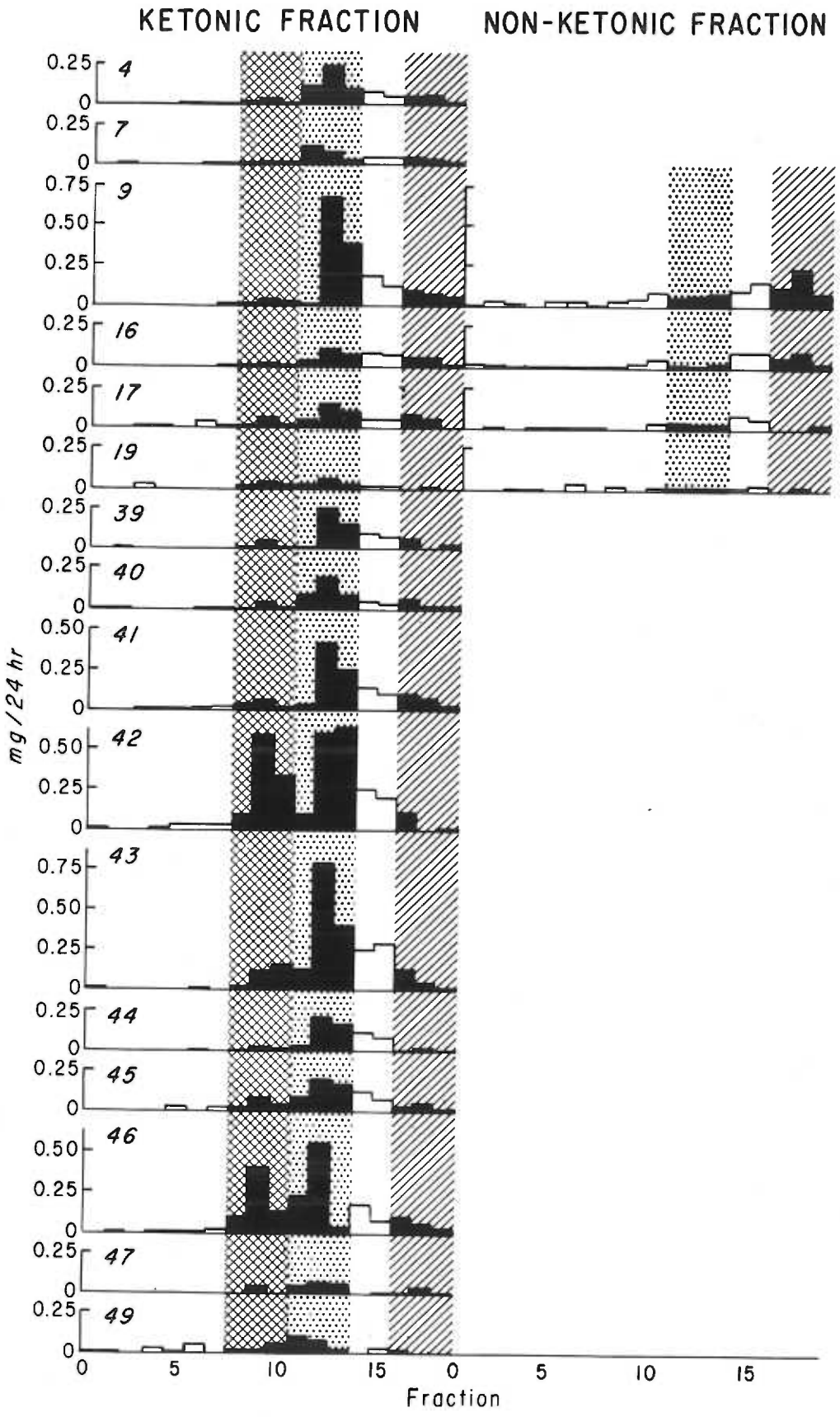

KETONIC FRACTION
NON-KETONIC FRACTION
4
7
9
16
17
19
39
40
41
42
43
44
45
46
47
49
mg/24 hr
Fraction

FIGURE NO. 19. Urinary 3β-OH-Δ^5-steroids from an anencephalic and a quadruplet pregnancy, respectively. Shaded areas correspond to accumulated fractions (see Table No. 8). Ordinates are in mg./24 h. Refer to the legend of Figure No. 16 for the explanation about the different accumulated fractions.

FIGURE NO. 20. Urinary 3β-OH-Δ^5-steroids from hirsute females. Shaded areas on each plot correspond to accumulated fractions (see Table No. 9). Ordinates are in mg./24 h. Refer to the legend of Figure No. 16 for the explanation about the different accumulated fractions.

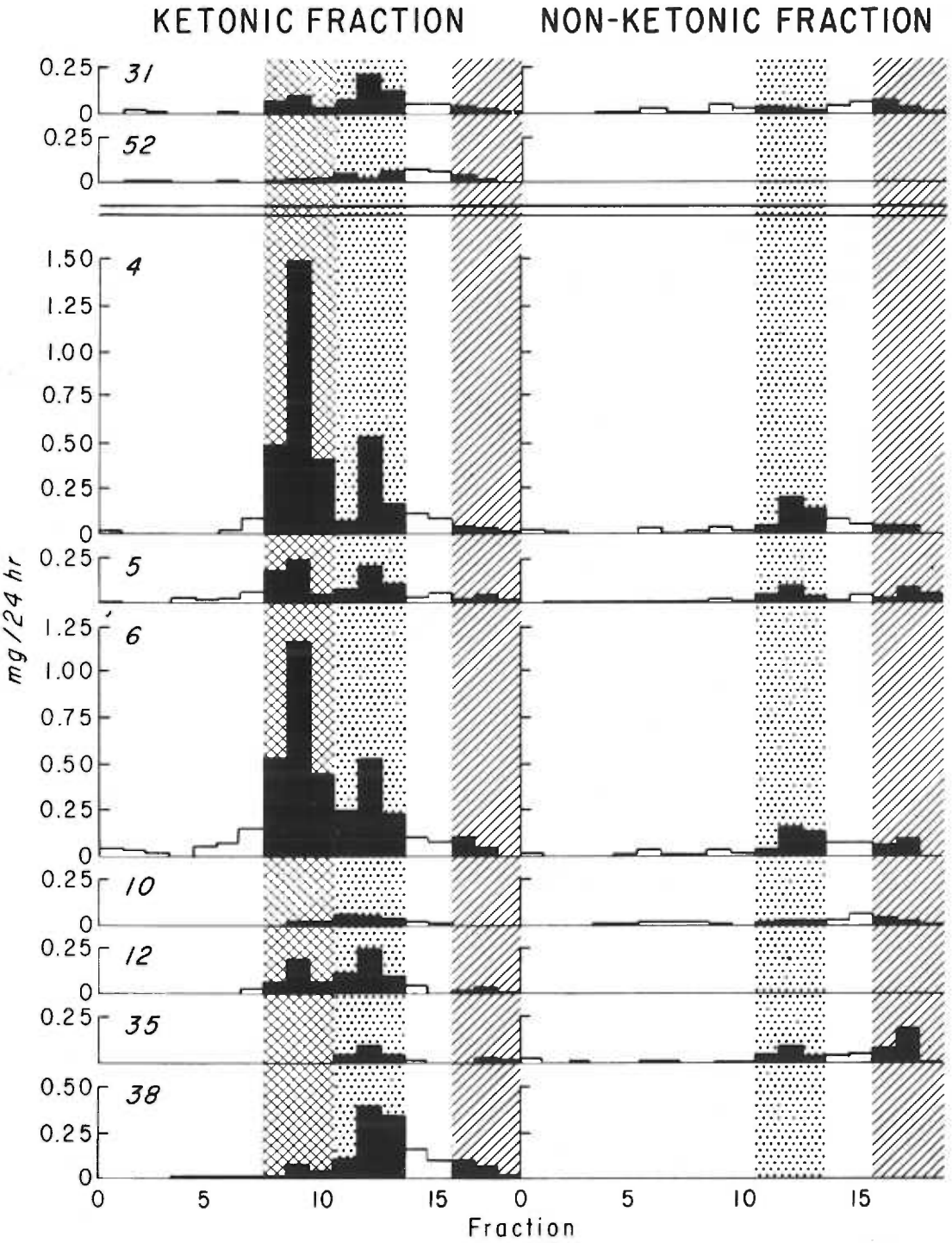

KETONIC FRACTION
NON-KETONIC FRACTION
31
52
4
5
6
10
12
35
38
mg/24 hr
Fraction

FIGURE NO. 21. Urinary 3β-OH-Δ^5-steroids from oligomenorrheic, nonhirsute females. Shaded areas correspond to accumulated fractions (see Table No. 10). Ordinates are in mg./24 h. Refer to the legend of Figure No. 16 for the explanation about the different accumulated fractions.

FIGURE NO. 22. Urinary 3β-OH-Δ^5-steroids from a boy with testicular feminization. Shaded areas correspond to accumulated fractions (see Table No. 11). Ordinates are in mg./24 h. Refer to the legend of Figure No. 16 for the explanation about the different accumulated fractions.

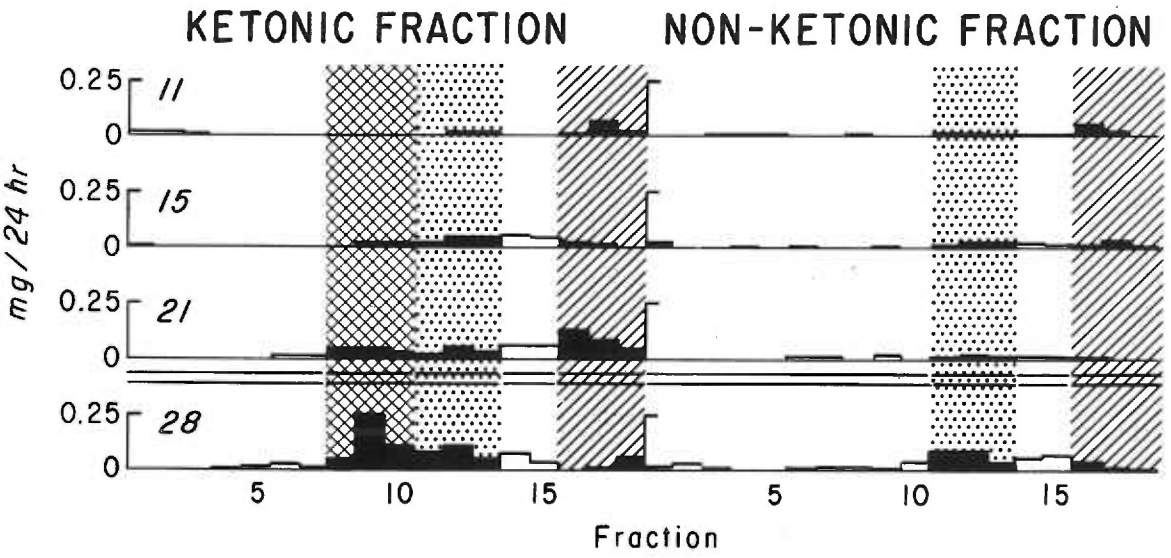

KETONIC FRACTION
NON-KETONIC FRACTION
mg/24 hr
0.25
0
11
15
21
28
5
10
15
5
10
15
Fraction

TABLE NO. 5. Urinary 3β-OH-Δ^5-steroids (mg./24 h.) from normal males.

TABLE NO. 6. Urinary 3β-OH-Δ^5-steroids (mg./24 h.) from normal females. Sampling was done with most subjects in the luteal phase of the menstrual cycle (see Appendix).

Subject No.	Ketonic Fraction			Nonketonic Fraction	
	#8 - 10 DHA	#11 - 13 16α-OH-DHA	#16 - 18	#11 - 13	#16 - 18
2	1.49	0.83	0.26	0.26	0.11
3	0.28	0.23	0.05	0.21	0.15
22	0.44	0.23	0.08	0.21	0.18
24	0.10	0.54	0.14	0.20	0.23
25	0.95	0.33	0.09	0.53	0.34
Ave. ± S.D.	0.65 ± 0.56	0.43 ± 0.25	0.12 ± 0.08	0.28 ± 0.14	0.20 ± 0.06

Subject No.	Ketonic Fraction			Nonketonic Fraction	
	#8 - 10 DHA	#11 - 13 16α-OH-DHA	#16 - 18	#11 - 13	#16 - 18
7	0.82	0.28	0.09	0.13	0.06
8	0.36	0.18	0.03	0.13	0.06
18	0.38	0.26	0.02	0.03	0.22
20	0.21	0.23	0.07	0.18	0.17
26	0.11	0.28	0.05	0.11	0
27	0.12	0.55	0.18	0.06	0.02
29	0.32	0.22	0.04	0.06	0.10
30	1.05	0.34	0.03	0.32	0.22
Ave. ± S.D.	0.42 ± 0.33	0.29 ± 0.12	0.06 ± 0.05	0.13 ± 0.09	0.07 ± 0.09

TABLE NO. 7. Urinary 3α-OH-Δ^5-steroids (mg./24 h.) from normal pregnancies. Sampling was carried out between 36 - 40 weeks with all subjects except Nos. 4 and 7 (see Appendix).

TABLE NO. 8. Urinary 3α-OH-Δ^5-steroids (mg./24 h.) from an anencephalic pregnancy at 31 weeks of pregnancy (Subject No. 31) and a quadruplet pregnancy of 34 weeks' duration (Subject No. 52).

Subject No.	Ketonic Fraction			Nonketonic Fraction	
	#8 - 10 DHA	#11 - 13 16α-OH-DHA	#16 - 18	#11 - 13	#16 - 18
4	0.09	0.47	0.12	—	—
7	0.03	0.22	0.08	—	—
9	0.09	1.10	0.24	0.18	0.38
16	0.04	0.22	0.15	0.07	0.20
17	0.10	0.31	0.15	0.10	0.04
19	0.09	0.12	0.01	0.06	0.01
39	0.05	0.41	0.08	—	—
40	0.06	0.40	0.11	—	—
41	0.11	0.69	0.21	—	—
42	1.03	1.35	0.13	—	—
43	0.32	1.33	0.20	—	—
44	0.06	0.45	0.04	—	—
45	0.12	0.46	0.08	—	—
46	0.67	0.83	0.21	—	—
47	0.05	0.17	0.04	—	—
49	0.09	0.21	0.01	—	—
Ave. ± S. D.	0.19 ± 0.27	0.55 ± 0.39	0.12 ± 0.08	0.10 ± 0.05	0.16 ± 0.17

Subject No.	Ketonic Fraction			Nonketonic Fraction	
	#8 - 10 DHA	#11 - 13 16α-OH-DHA	#16 - 18	#11 - 13	#16 - 18
31	0.17	0.39	0.09	0.10	0.11
52	0.05	0.12	0.11	—	—

TABLE NO. 9. Urinary 3β-OH-Δ^5-steroids (mg./24 h.) from hirsute females (see Appendix for clinical data).

TABLE NO. 10. Urinary 3β-OH-Δ^5-steroids (mg./24 h.) from oligomenorrheic, nonhirsute females (see Appendix for clinical data).

TABLE NO. 11. Urinary 3β-OH-Δ^5-steroids (mg./24 h.) from a 16-year-old boy with testicular feminization.

Subject No.	Ketonic Fraction			Nonketonic Fraction	
	#8 - 10 DHA	#11 - 13 16α-OH-DHA	#16 - 18	#11 - 13	#16 - 18
4	2.38	0.78	0.09	0.39	0.11
5	0.46	0.40	0.08	0.17	0.18
6	2.16	1.01	0.15	0.34	0.14
10	0.04	0.13	0.01	0.05	0.08
12	0.32	0.46	0.06	0.14	0.03
35	0	0.16	0.03	0.15	0.27
38	0.10	0.53	0.17	—	—
Ave. ± S.D.	0.78 ± 1.29	0.50 ± 0.31	0.08 ± 0.02	0.21 ± 0.04	0.14 ± 0.03

Subject No.	Ketonic Fraction			Nonketonic Fraction	
	#8 - 10 DHA	#11 - 13 16α-OH-DHA	#16 - 18	#11 - 13	#16 - 18
11	0	0.03	0.10	0.03	0.05
15	0.02	0.08	0.03	0.06	0.06
21	0.11	0.09	0.25	0.06	0
Ave. ± S. D.	0.04 ± 0.02	0.07 ± 0.03	0.13 ± 0.04	0.05 ± 0.02	0.04 ± 0.01

Subject No.	Ketonic Fraction			Nonketonic Fraction	
	#8 - 10 DHA	#11 - 13 16α-OH-DHA	#16 - 18	#11 - 13	#16 - 18
28	0.39	0.23	0.06	0.20	0.08

in 5 males was 0.65 ± 0.56 mg. per 24 hours (range 0.1 - 1.49 mg.); the mean value for 16α-OH-DHA (fractions 11 - 13) was 0.43 ± 0.25 mg. per 24 hours (range 0.23 - 0.83 mg.). Some more polar Pettenkofer chromogens consistently appeared in small quantities in the chromatograms of the ketonic fraction but no attempts for identification were made.

The nonketonic fraction of each male's urine contained Pettenkofer positive material that was eluted into two peaks. One of the peaks (fractions 11 - 13) consisted of compounds which had paper chromatographic characteristics (See Methods) similar to pure androstenediol. The urinary content represented by the material in this peak was 0.28 ± 0.14 mg. per 24 hours (range 0.2 - 0.53 mg.). The second peak of more polar material (fractions 16 - 18) in the nonketonic fraction appeared in each urine with a mean value of 0.20 ± 0.06 mg. per 24 hours (range 0.11 - 0.34 mg.) but no identification procedures were attempted.

Among 8 normal females (ages 19 - 30 years) whose urine was sampled mainly in the luteal phase of the menstrual cycle, 5 excreted more DHA than 16α-OH-DHA. The mean urinary steroid content of the entire group was 0.42 ± 0.33 mg. per 24 hours (range 0.1 - 1.05 mg.) of DHA and 0.29 ± 0.12 mg. per 24 hours

(range 0.18 - 0.55 mg.) of 16α-OH-DHA. The unidentified polar ketonic material appeared in small variable quantities in each urine.

The nonketonic fraction from normal females usually exhibited two peaks. The chromogens that had chromatographic characteristics as androstenediol represented a mean excretion value of 0.13 ± 0.09 mg. per 24 hours (range 0.03 - 0.32 mg.). Smaller amounts of the more polar nonketonic material were present in all except one urine.

2. Pregnancies: Urine collected from 16 women (ages 21 - 41 years) late in their clinically normal pregnancies had more 16α-OH-DHA than DHA. The mean excretion value for 16α-OH-DHA was 0.55 ± 0.39 mg. per 24 hours (range 0.12 - 1.35 mg.) and was 0.19 ± 0.27 mg. per 24 hours (range 0.03 - 1.03 mg.) for DHA. The unidentified polar ketonic material was demonstrated in smaller variable quantities. Significant amounts of Pettenkofer positive compounds with chromatographic mobilities between 16α-OH-DHA and the most polar material always appeared. No attempts for identification were made. The amounts of nonketonic components measured in 4 specimens were so small that exploration of nonketonic fractions in others did not seem promising.

Urine specimens from two abnormal pregnancies

were analyzed. In the 32nd week of a pregnancy with an anencephalic fetus, 0.17 mg. of DHA and 0.39 mg. of 16α-OH-DHA were excreted in 24 hours. Other polar ketonic and nonketonic compounds were recognized in small quantities. The excretion rate of DHA and 16α-OH-DHA in the urine during the 8th month of pregnancy from a subject who had quadruplets was 0.05 mg. and 0.12 mg. per 24 hours, respectively. The nonketonic fraction was not measured.

3. Hirsute females: In 7 hirsute females, the excretion values for DHA varied from 0 to 2.38 mg./24 h. and for 16α-OH-DHA, from 0.13 to 1.01 mg./24 h. The respective means were 0.78 ± 1.29 and 0.50 ± 0.31 mg. The polar ketonic material (fractions 16 - 18) appeared in 0.08 ± .02 mg. per 24 h. quantities in the range 0.01 - 0.17 mg. but did not represent distinct peaks. In four of the extracts there was a significant amount of unidentified ketonic material that was eluted from the column after 16α-OH-DHA.

The nonketonic fraction from 4 urine extracts was quantitated. Two peaks of Pettenkofer positive material were always present, the first one having the chromatographic mobility of androstenediol. The mean excretion value of that peak present in fractions 11 - 13 was 0.21 ± 0.04 mg./24 h. (range 0.05 - 0.39 mg.). The more

polar nonketonic peak contained in fractions 16 - 18 had 0.14 ± 0.03 mg. mean value and the range 0.03 - 0.27 mg. No significant amount of Pettenkofer material was measured outside the fractions comprising each peak. The urine from Subject No. 35 contained no measurable DHA but had the most prominent (0.27 mg./24 h.) polar nonketonic peak among the hirsute females. Subjects No. 4 and 6 excreted large amounts of DHA (2.38 and 2.13 mg./24 h.). The same individuals also excreted more 16α-OH-DHA and nonketonic material in fractions 11 - 13.

4. Oligomenorrheic, nonhirsute females: Urine from three young adult subjects who were not hirsute but experienced irregular and infrequent menses contained minimal amounts of any 3β-OH-Δ^5-steroids (see Fig. 8). Excretion rates for DHA ranged from 0 - 0.11 mg. per 24 hours with an average value of 0.04 mg. The corresponding values for 16α-OH-DHA were a range from 0.03 - 0.09 mg. per 24 hours and a mean value of 0.07 mg. Other Pettenkofer positive material was present in minimal amounts.

5. Testicular feminization: The 24-hour urine specimen from a 16-year-old boy with testicular feminization contained 0.39 mg. of DHA, 0.23 mg. of 16α-OH-DHA and 0.2 mg. of nonketonic material in fractions 11 - 13.

IV. DISCUSSION

The development of a simplified method for the chemical quantitation of urinary 3β-OH-Δ^5-steroids arose from our proposal to investigate applications to clinical medicine of the principle stated in the Introduction. The various analytical procedures finally incorporated into the method were chosen with regard to the practicability of their use in addition to the paramount consideration of analytical reliability. After the total method was assembled, we established its reliability and analyzed some urine specimens from several types of clinical subjects. Comparisons with published data are possible in some instances but not in others, where our data represent the only information available.

There are few methods for the systematic analysis of 3β-OH-Δ^5-steroids in biological samples since most determinations of this group of compounds have relied upon unrelated ring-D chemical properties or, if the 3β-ol-Δ^5-configuration was the basis for quantitation, the total group rather than individual members was measured. Moreover, the design of methods for isolation and identification procedures frequently do not adapt to the requirements of numerous, repetitive determinations. The available methods devised to detect one or more individual steroids of this group involve complicated extractions, separations or chemical transformations for quantitation which, undoubtedly, add to their resolution and specificity but also to their losses and time requirements (21, 27, 85, 104, 112).

Hydrolysis

Since urinary 3β-OH-Δ^5-steroids exist principally as sulfate esters, and the technical problems associated with the analysis of steroid conjugates are presently unresolved, hydrolytic procedures to secure free steroids are mandatory. Comparisons of several methods of hydrolysis including hot acid, boiling at a neutral pH, enzymic and solvolytic procedures suggest that the least artifact formation and highest rate of hydrolysis can be expected in a reasonable time using the solvolytic procedure described by Burstein and Lieberman (10, 11, 15, 36, 57). Although most of the comparisons have been made with DHA, the additional deleterious effects of hot acid hydrolysis upon the α-ketolic moiety of 16α-OH-DHA has been recognized (36). The recoveries of steroids reported here after extraction and solvolysis in the order of 90 percent agrees with other reported rates (15, 10).

Girard Separation

We established that chemical purification of the urinary fractions was essential before submitting them to column chromatography. Without purification, urinary pigments and unidentified lipid-like materials were visible on the column and caused a turbid solution in the colorimetric reaction procedure. This has been the experience of others (11, 57) and introduction of a chemical purification circumvented this problem. The addition of Girard's reagent T permits the formation of water-soluble hydrazones with steroid

ketones. The instability of the product is exploited by extraction of the nonketonic steroids from an alkaline solution and the ketonic steroids from the acidified solutions. Losses of steroids due to the Girard purification, usually estimated up to 15%, were about 20 percent in this study (105).

Column Chromatography

The separation of the steroids was accomplished by partition chromatography. The discontinuous gradient-elution technique is a compromise alternative to the laborious methods necessary to insure reproducible gradients in the solvent composition of the mixtures employed for separation and the effect is similar. The procedure adopted had been particularly useful for the separation of very polar C_{21} metabolites and could be expected to have the resolution capacity for the hydroxylated steroids that were likely to be found in the urine specimens of the proposed study. Adequate resolution of DHA and 16α-OH-DHA was demonstrated using pure compounds. Resolution of those compounds in urinary extracts was likewise adequate although chemical homogeneity in the peak corresponding to 16α-OH-DHA was unproven, and unlikely in view of the paper chromatographic separation of additional small amounts of Pettenkofer positive material that had an R_f of 0.94 in ketonic fraction 12. Yet the majority of the Pettenkofer positive material in that fraction also gave a blue tetrazolium positive test denoting an α-ketol, which is the expected result for 16α-OH-DHA.

Colorimetry

Popularly used colorimetric methods for quantitation that are reasonably specific for this class of steroids depend upon the formation of a colored complex with 2-furaldehyde or ethanol in the presence of concentrated sulfuric acid. The precise reaction mechanisms have not been demonstrated but it is likely that the complex appears as the result of protonation of C-6 in ring B of the steroid nucleus with the formation of a carbonium ion capable of temporary association with an atom, probably oxygen, in the furaldehyde or ethanol.

An investigation of the features that caused cholic acid to give a strongly positive Pettenkofer reaction caused Munson, et al. (83) to propose a successful modification and to generalize that unsaturation in ring B (or group that could give ring B unsaturation) and a hydroxyl group or double bond in ring A are essential structural requirements for a positive reaction. All but 3β-hydroxy-Δ^5-androstene, of the 16 compounds that gave a positive result, had a functional group elsewhere in the molecule. Thus cholesterol gave a negative test but 3β-OH-Δ^5-lithocholenic acid, a positive one. All of the C_{21} and C_{19} steroids with the 3β-OH-Δ^5-configuration that we expected in urine gave positive modified Pettenkofer reactions but with varying extinction coefficients. They made the following approximations about color intensity produced by different functional groups based on 56 steroids tested:

C-20 ketone < C-17 ketone

ketone > hydroxyl

2 ring D hydroxyls ≲ C-20 ketone

C-20 or 21 ketol increases intensity

When the molar extinction coefficients of some 3β-OH-Δ^5-steroids [data from Munson, et al. (83) and Fotherby (33)] are related percentage-wise to that of DHA, the following values are obtained:

Steroid	Percent
DHA	100
DHA-sulfate (K salt)	105
16α-OH-DHA*	69
androstenediol	25
androstenetriol	75
pregnenolone	78
pregnenediol	< 20

* Fotherby (33)

The failure of the modified Pettenkofer reaction to obey Lambert-Beer's Law is well known and represents its major limitation for application to estimate 3β-hydroxy-Δ^5-steroids in biological materials. In the range 0 - 10 μg. of DHA per ml. of reaction mixture, the extinctions reasonably approximate linearity but failure to do so at larger concentrations can only be overcome by interpolation using several concentrations of standards. In practice, critical estimates of anticipated concentrations are made so that their extinctions

will correspond to the optimal area of the curve. Day to day variations in the reproducibility of the reaction are 5 - 10 percent but this variation is reduced by the application of Allen's correction which decreases the extinctions at maximum absorbance by amounts contributed by nonspecific chromogens measured at adjacent points of the spectrum. The modification proposed by Inagaki escaped our attention until after the present work was initiated but our preliminary experiments confirm that improvements in the chromogenic response can be achieved (57).

The Oertel-Eik-Nes reaction for 3β-OH-Δ^5-steroids gave distinctive spectra for several steroids tested; however the wave length of maximal absorbance differed for some members of the group and also varied with time. The spectra are distinctive enough to be useful as identification criteria (10, 111) in qualitative analysis but the reaction has limited value for quantitation of 3β-OH-Δ^5-steroids as a group. This is regrettable since the chromogenic response is a linear one and would have facilitated the colorimetry.

Reliability

The evaluation of the sensitivity and precision of the colorimetry are not strictly comparable to the data available since Fotherby determined the precision as 0.06 mg./24 hrs. (36) and precision determined in this study was 0.00024 mg. at a 0.03 mg. level. At the level of the mean excretion value for DHA in men (0.65 mg./24 h.) our calculated precision per 24 hrs. is about 0.052 mg.

Similarly, the sensitivity was 0.00048 mg. out of 0.030 mg. and the calculated sensitivity per 24 hrs. was 0.104 mg. compared to 0.25 mg. estimated by Fotherby from recovery experiments data.

The specificity of the method relies upon the combined specificities of the hydrolytic, Girard separation, chromatographic and colorimetric procedures. The specificity of the Pettenkofer reaction was reasonably well established by Munson, et al., however, the procedures that we combined were tested for specificity and accuracy by addition of free and/or conjugated pure DHA and 16α-OH-DHA to water and urine. The combined recoveries were smaller in urine than in water with the range 68 - 78 percent. Up to 20 percent losses could be ascribed to the Girard separation. Therefore the recoveries obtained in the present experiments compare nearly identically to those of Fotherby; without any chemical purification procedure, he recovered up to 87 percent of added DHA-sulfate from urine. The recovery of only 68 - 79 percent of the added steroid in these experiments is not optimal; however it was reproducible in 4 water and urine extracts and is considered permissible.

Comparison of Results: Normal Subects

The range of values of DHA excreted by normal males and females obtained by the method described are in an intermediate range in comparison with those obtained by other procedures (see Table No. 3). The average of the values for DHA excretion in the present small group of males is less than the 1.2 mg./24 h. re-

ported by Fotherby but within the range of values he found (36). For females, the 0.42 mg./24 h. average of the present study compares with 0.5 mg./24 h. determined by Fotherby and the respective ranges of comparative values are 0.11 - 1.05 and 0.0 - 2.0 mg./24 h. Wilson (112) determined a range of 0 - 0.9 mg./24 h. in normal females although the time for sampling in the menstrual cycle was not reported in either paper.

Comparison of excretion values of 16α-OH-DHA is not possible for lack of reported results but Fotherby, et al. (33), crystallized 0.05 - 0.15 mg./l. of 16α-OH-DHA from urine of human males. Inspection of the range of determined values discloses a more narrow range than with DHA values and no tendency for a constant ratio DHA/16α-OH-DHA. In both men and women there is approximately one-third less 16α-OH-DHA than DHA; however the uncorrected results for 16α-OH-DHA are underestimates. Introduction of a correction factor for the lesser molar extinction coefficient of 16α-OH DHA (65% of DHA) would reemphasize its quantitative contribution.

The most polar ketonic and nonketonic fractions were not identified but apparently significant amounts of Pettenkofer positive material are regularly present in those fractions. One might speculate that the ketone is a C_{21} compound as 17α-OH-pregnenolone or a 7-oxygenated disubstituted C_{19} metabolite, and the polar nonketone could be a member of either the C_{21} or C_{19} series such as pregnenetriol, androstenetriol or the 7-hydroxylated ring-D glycol. It may be significant that a "cold Munson" reaction* was never observed.

*"cold Munson" reaction denotes a typical color development before heating and considerable fading after heating. It resembles the Lifschütz reaction for 7-OH-Δ^5-steroids (70).

It appears on the basis of paper chromatographic data presented in "Results" that androstenediol is present in tubes 11 - 13 of the nonketonic fractions. The mean values for men and women make it the third most abundant member of the group and its excretion rate would be quadrupled if the molar extinction correction factor for androstenediol was introduced (25% of DHA). Fotherby and Wilson found up to 0.5 mg./24 h. of urinary androstenediol in men.

Correlations in Pregnancy

The reversed ratio DHA/16α-OH-DHA in pregnancy urine was totally unexpected. The urinary content of DHA during pregnancy was known to be reduced (14) although no physiological significance was apparent. We find no recorded values for urinary 16α-OH-DHA in pregnancy urines. The average value was nearly double that found in nonpregnancy urines and even greater than the amounts excreted by normal males. The increase during pregnancy could be due to elevated secretion rates by maternal adrenal gland or ovary or facilitated hepatic metabolism of appropriate compounds. Maternal adrenal or ovarian hyperactivity is unlikely (28) and rates of hepatic metabolism of steroids in pregnant women have not been explored. However 16α-hydroxylation is a prominent transformation in fetal hepatic and perhaps adrenal gland tissue. The increment of increase in the urine could result from a "leak" through the placenta from the abundant amounts of 16α-OH-DHA present in the fetal circulation although

the maternal plasma values are identical in peripheral blood and in effluent blood from the utero-placental circulation (24, 25). No fetal weight or sex correlations are possible with 16α-OH-DHA excretion.

The decreased urinary content of DHA in pregnancy might be explained by transformation of circulating DHA into estrone by the placenta. Aromatization of DHA, androstenediol, and 16α-OH-DHA to estrone, estradiol and to 16α-OH-estrone (and thereafter, to estriol), respectively, has been demonstrated in studies in vitro (28). The same reaction would therefore tend to reduce rather than increase urinary 16α-OH-DHA, assuming that enzyme affinities for the substrates were identical. An alternative explanation is that urinary 16α-OH-DHA reflects to some degree, increased concentrations circulating in blood as a result of relative insufficiency in its conversion to estrogen. Plasma values of 16α-OH-DHA are usually lower, however, than DHA values during pregnancy (25). These relationships warrant additional investigation. The other 3β-OH-Δ^5-steroids that were quantitated appear to fluctuate with 16α-OH-DHA. Although not unique in pregnancy urines, the ketonic material exhibiting polarity properties between those of 16α-OH-DHA and the most polar Pettenkofer positive material are present in significant quantities. It further emphasizes a need to examine pregnancy urines more closely.

Some of the 3β-OH-Δ^5-steroids were measured in two abnormal pregnancies to determine whether this group of compounds are excreted in abnormal amounts. We have data showing that the 16α-

OH-DHA content in the fetal circulation of anencephalic fetuses is about 1/10 of normal values (24). This agrees with the decrease of estriol excreted in those pregnancies and supports, albeit indirectly, a precursor role by 16α-OH-DHA for estriol (28). The urinary excretion of DHA and 16α-OH-DHA from the anencephalic pregnancy compares with normal term pregnancies as well as two normal pregnancies sampled similarly in the 28 - 30th week (see appendix). This data suggests that the inverted ratio of DHA/16α-OH-DHA in pregnancy is not related to "leakage" through the placenta. A minimal urinary content of DHA and 16α-OH-DHA was found in the quadruplet pregnancy where the relative fetal weight was more than triple the expected normal fetal weight at 28 - 30 weeks gestation. A correlation of increased urinary estriol and normal twin pregnancies led to the present effort to determine whether urinary 16α-OH-DHA was also related to fetal weight. The low value obtained is additional evidence for an insignificant quantitative contribution to maternal urinary 16α-OH-DHA by the fetus. The very low values may indicate that the placenta actively converts circulating DHA in the mother to other products, probably estrone and estradiol, with a 'net reduction of urinary DHA and probably other circulating 3β-OH-Δ^5-steroids (28).

Hirsute Females

The average and the range of excretion values of both DHA and 16α-OH-DHA were larger in hirsute females than in either normal males or nonpregnant females. A comparison of reported values

of DHA excretion in Table No. 3 and the data from this study shown in Table No. 7 demonstrates that the range is wide and the present results are similarly variable. The DHA/16α-OH-DHA ratio was not constant although when DHA concentrations were high, values of other fractions tended to increase too. The degree of hirsutism of individual subjects (see Appendix) did not correlate with the urinary values for DHA. This has also been the observation of other investigators (14). Subject No. 35 had sclerocystic disease of her ovaries and the most marked hirsutism but she also had the lowest excretion of 3β-OH-Δ^5-steroids of all of the hirsute subjects. The excretion values in Subjects No. 4 and 6, whose diagnosis was based on clinical data, were roughly similar yet the intensity of their symptoms was greatly different. These facts are consistent with current concepts of alternative androgen metabolism via compounds that have the 3β-OH-Δ^5-configuration (77). Evidence has been offered suggesting that some subjects with clinical expressions and morphologic characteristics of disease that are indistinguishable may have very dissimilar plasma or urinary products of androgen metabolism. Recent demonstrations of the ovarian secretion of testosterone and androstenedione in the 3-keto-Δ^4-group and DHA in the 3β-OH-Δ^5-group by normal women permit coherent conclusions about function from observations of altered androgen metabolism in the masculinizing endocrinopathies (40, 99). Urinary testosterone is usually measured in concentrations near 0.100 mg./24 h. in normal young men and near 0.008 mg./24 h. in normal females. Concentrations in the urine and the plasma of hirsute women range widely but are often in the male range or even

higher. Abnormal rates of enzymic transformations in ovarian tissue (Δ^5-3β-hydroxysteroid dehydrogenase; 17α-hydroxylase and aromatization) have been postulated as the responsible factors in the development of the hirsutism-related endocrinopathies (77). The stimulatory or inhibitory controls of pituitary hormones on the ovary, apparent in fluctuations of urinary steroid metabolites, probably function by altering enzyme kinetics to establish the cyclical pattern. Given the adrenal and ovary as sources for androgen precursors, at least two known androgen metabolic pathways responsive to cyclic pituitary hormones, and hepatic metabolism of circulating metabolites, all presently-conceived investigations to determine the diseased organ in patients with the various endocrinopathies are complex and often inconclusive.

The recent determination of production rates of some compounds such as DHA adds another parameter to our understanding of steroid metabolism (107). Rates of 12 - 24 mg./24 h. estimated for DHA production in normal women emphasize the quantity of steroids produced. But even the comparison of production and excretion rates leaves unanswered the rates of transformation to other steroidal products, as estrogens for example, or nonsteroidal degradation products such as CO_2 or other metabolites. Conceptual inadequacies of the biologic properties of many steroid compounds in humans, the influence(s) of conjugation and tissue responses, the hair follicle, for example, have prevented greater progress in this area of research.

Oligomenorrheic, Nonhirsute Females

The uniformity of the reduced excretion of 3β-OH-Δ^5-steroids in 3 cases of hypomenorrhea was surprising. Each of the subjects in this group had clinical effects of hypoestrogenism as well as infrequent or absent menses yet all exhibited evidences of normal feminine development. A lack of hirsutism or other symptoms or findings of hyperandrogenism and 17-KS within the normal range contrasts this group with the hirsute group. Failure to excrete usual amounts of steroids and to experience normal sexual function could result from pituitary, adrenal or ovarian malfunction. Application of some dynamic tests might answer the diagnostic problem.

Testicular Feminization

Finally, the excretion pattern of 3β-OH-Δ^5-steroids in the boy with testicular feminization was not distinctive and was compatible with either sex. No specific biochemical defects have been demonstrated in the testes of patients with testicular feminization. Failure of growth of axillary hair, and often pubic hair, even with therapeutic doses of androgens suggests that the defect is not limited to steroid biosynthesis and metabolism.

This study adds to accumulating data that emphasizes the importance of 3β-OH-Δ^5-steroids in human metabolism. Estimated production rates of DHA in milligram quantities are corroborated by a plasma concentration greater than any other steroid measured in plasma. It is the third most abundant urinary neutral 17-KS and

the precursor for the most abundant ones, androsterone and etiocholanolone (14). Results of the present analyses establish 16α-OH-DHA as a quantitatively significant urinary 3β-OH-Δ^5-metabolite in normal subjects. Moreover, deviations from "normal" values of DHA and 16α-OH-DHA occur frequently in women with 'masculinizing' syndromes or menstrual irregularities. These observations suggest a physiological role by DHA and 16α-OH-DHA, and perhaps other of the incompletely identified 3β-OH-Δ^5-steroids, in the metabolism of androgens and estrogens in men and women. By comparison, in pregnancy only a precursor role for these compounds can be postulated but no physiological significance is known for the huge amounts of urinary estrogens that represent transformation products of 3β-OH-Δ^5-steroid metabolism.

On the basis of these observations, I think that valuable information about androgen metabolism in pregnancy could be gotten by comparative studies of urinary, maternal and fetal plasmatic 3β-OH-Δ^5-steroids. Also, studies of the physiological relationship between this group of steroids and estrogens will probably be fruitful, not only in pregnancy but also in the fluctuations of individual estrogens that occur during normal menstrual cycles. A challenging area for investigation is the steroid 16α-hydroxylation mechanism, its organ sites and responses, if any, to pituitary trophic hormones. Finally, the method described can be useful in evaluations of 'masculinizing' endocrinopathies but cannot give decisive information by itself for either diagnosis or therapy. Coupled with additional evaluations of androgen metabolism, such as plasma or urinary testosterone and individual urinary neutral

17-KS and estimates of at least the classic estrogens (estrone, estradiol and estriol), reasonable estimates could be made of certain steroid parameters related to reproductive function.

V. SUMMARY AND CONCLUSIONS

1. A method has been developed that is useful for the analysis of dehydroepiandrosterone (DHA) and 16α-hydroxydehydroepiandrosterone (16α-OH-DHA) in urinary extracts. The accumulated fractions may also include other Pettenkofer positive material with similar chromatographic mobilities. Other 3β-OH-Δ^5-steroids were observed but their individual fractions were not identified as rigorously.
2. The method comprises ethyl acetate extraction and solvolysis, separation and purification with Girard's reagent T, column chromatography with silica gel, and colorimetry with the modified Pettenkofer reaction.
3. The analytical reliability of the method has been evaluated and can be described by a sensitivity of 0.1 mg./24 h. and a precision of 0.05 mg./24 h. Accuracy has been evaluated by consistent recoveries of 68 - 78 percent of pure steroids introduced into water and urine specimens. The specificity data determined with a few compounds agreed with comprehensive data available in the literature.
4. Excretion values of DHA in 5 normal men ranged from 0.1 - 1.49 mg./24 h. The average was 0.65 ± 0.56. Uncorrected values for 16α-OH-DHA were 0.23 - 0.83 mg./24 h. and 0.43 ± 0.25 average.
5. The average values for 8 normal women for urinary DHA was 0.42 ± 0.33 mg./24 h. (range 0.11 - 1.05) and for 16α-OH-

DHA, was 0.29 ± 0.12 mg./24 h. (range 0.18 - 0.55).

6. Women in late pregnancy excreted 0.19 ± 0.27 mg./24 h. of DHA (range 0.03 - 1.03) and 0.55 ± 0.39 mg./24 h. of 16α-OH-DHA (range 0.12 - 1.35). The excretion pattern in an anencephalic pregnancy was similar to that in normal pregnancies but the rates were reduced in a quadruplet pregnancy.
7. Some hirsute women excreted more DHA and 16α-OH-DHA than normal men or nonpregnant women. The respective average values for 7 women were 0.78 ± 1.29 and 0.50 ± 0.31 mg./24 h. The range of values for them was 0 - 2.38 and 0.13 - 1.01 mg./24 h.
8. Three nonhirsute, oligomenorrheic women excreted minimal amounts of any Pettenkofer positive material. Values obtained from a boy with testicular feminization were not distinctive.
9. The results of this study command a new appreciation for the quantitative importance of 16α-OH-DHA in normal androgen metabolism of both sexes.
10. Every pregnancy urine analyzed contained more 16α-OH-DHA than DHA. This is an opposite excretion pattern to the one found in men and some nonpregnant women, and has gone unrecognized previously.
11. Excretion values for DHA and 16α-OH-DHA varied greatly among the hirsute subjects and were not related to the intensity of symptoms.
12. Abnormal excretion patterns of 3β-OH-Δ^5-steroids by women with menstrual irregularities, with or without hirsutism, suggest that estrogen metabolism and the 3β-OH-Δ^5-steroids are closely related.

VI. APPENDIX

Descriptive clinical data about similar groups of normal subjects or patients is presented below. Hospital records, when applicable are identified in parentheses after the subject's study number. The determinations made by the Clinical Laboratories of the University of Oregon Medical School, Hospitals and Clinics are marked with an asterisk (*). They include neutral 17-ketosteroids (22), 17-OH corticosteroids (17-OHCS) [a few values obtained by this method are indicated by (xx)] (84), and Porter-Silber steroids (91).

NORMAL MALES

Subject No.	Age
2	31
3	21
22	35
24	19
25	24

NORMAL FEMALES

Subject No.	Age	Day of Menstrual Cycle
7	21	16
8	29	11
18	19	20
20	28	30
26	24	20
27	23	15
29	25	17
30	30	18

NORMAL PREGNANCIES

Subject No.	Age	Gestation Duration (wks)	Infant Sex	Infant Weight (g.)
4	26	30	Male (M)	—
7 (33-50-70)	22	28	Female (F)	—
9	22	37	M	—
16 (28-96-39)	22	37	F	—
17 (29-02-74)	21	36	F	—
19 (31-38-62)	20	37	M	—

Subject No.	Age	Gestation Duration (wks)	Infant Sex	Infant Weight (g.)*
39 (17-94-55)	32	37	F	2974
40 (31-82-15)	25	39	F	4394
41 (29-71-85)	28	39	M	3546
42 (32-68-45)	37	39	M	2946
43 (27-71-33)	22	38	M	2946
44 (29-74-31)	29	38	M	3602
45 (32-48-71)	24	40	F	2904
46 (32-94-56)	34	39	M	3517
47 (11-19-92)	41	39	F	4080
49 (31-72-01)	22	41	M	2974

*Weight given if delivery occurred within 24 hours after urine sampling.

ABNORMAL PREGNANCIES*

31	22	31	F (anenceph.)	1459
52	19	34	F (quadruplets)	5192 (total)

*both were in labor.

HIRSUTE FEMALES

Subject No. 4: A 26-year-old patient had menarche at 10 years of age followed by regular menses for 6 years. Thereafter, she had irregular menses for 3 years and finally amenorrhea for 4 years. She had a feminine appearance with minimal hirsutism. After cyclic estrogen-progesterone therapy, given empirically, she conceived one week prior to the first urine study.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Week(s) of pregnancy		
	1	22	30
DHA	2.38	0.51	0.09
16α-OH-DHA	0.78	0.85	0.47
polar ketonic	0.09	0.14	0.12
nonpolar nonketonic	0.39	0.16	—
polar nonketonic	0.11	0.27	—
Time	May, 1963	Sept., 1963	Nov., 1963

Subject No. 5 (27-60-44): A 21-year-old patient had normal menses until a normal pregnancy ensured 4 years ago. Thereafter, she had one mensus followed by 6 months of amenorrhea. Acute abdominal symptoms culminated in surgical removal of an ovary which contained a large follicular cyst. Thereafter, progressive facial and body hirsutism and infertility were noticed despite regular menses. Urinary neutral 17-KS and 17-OHCS were normal.

She returned in 1963 with the same complaints, a slightly enlarged ovary and moderate facial and body hirsutism. Her endocrine studies were interrupted by unrelated circumstances. Her diagnosis was not established.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baseline
17-KS *	25.2
Porter-Silber *	1.9
DHA	0.46
16α-OH-DHA	0.40
polar ketonic	0.08
nonpolar nonketonic	0.17
polar nonketonic	0.18
Time	April, 1963

Subject No. 6 (31-14-44): A 19-year-old student with a 3-year history of intermittent poly- and oligomenorrhea had increasing facial and body hirsutism. Endocrine laboratory studies follow.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baselines		Dexamethasone 2nd day of 2 mg. Q.D.	ACTH I.V. 2nd day 25 u. in 8 h.	Baseline	Hydrocortisone 40 mg. Q. D.	Cortisone 50 mg. Q. D.	Prednisone 10 mg. Q. D.
17-KS*	24.4	—	5.9	9.8	21.4	17.5	11.8	12.3
Porter-Silber*	(14.8)	4.0	1.5	16.7	9.1	7.9	10.8	7.6
pregnanetriol	—	—	—	—	4.6	—	—	—
DHA	2.16	1.36	0.16	0.71	1.15	0.04	—	—
16α-OH-DHA	1.01	0.95	0.20	0.54	0.44	0.14	—	—
polar ketonic	0.15	0.20	0.01	0.12	0.07	—	—	—
nonpolar non-ketonic	0.34	0.15	0.06	0.06	0.25	—	—	—
polar non-ketonic	0.14	0.28	0.15	0.15	0.27	—	—	—
Time	March, 1963 →		August, 1963 →		Oct., 1963	Dec., 1963		Feb., 1963

With prednisone therapy, the patient has resumed menstruation and ovulation.

Subject No. 10 (25-66-91): A 23-year-old patient had a normal pregnancy and an abortion with normal menses for 1 year thereafter. About a year and one-half before this investigation she began having fewer menses, a 60-pound weight gain and an increased growth of facial, arm and body hair. She failed to return to continue the investigation.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baseline	Baseline	Baseline
17-KS *	—	27.3	7.8
Porter-Silber *	13.3	—	11.7
DHA	—	—	0.03
16α-OH-DHA	—	—	0.23
polar ketonic	—	—	0
nonpolar nonketonic	—	—	0.09
polar nonketonic	—	—	0.08
Time	April, 1963 ⟶		May, 1963

Subject No. 12 (17-98-17): A 21-year-old patient noted the onset of facial and body hirsutism during her last pregnancy one year before this investigation. At a repeat cesarean section, her ovaries looked normal. Thereafter, she complained of oligomenorrhea and progressive hair growth. This patient failed to continue the investigation for reasons unknown to me.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baseline	Baseline	Baseline
17-KS *	18.6	16.9	21.1
Porter-Silber *	3.8	2.7	4.5
DHA	—	0.32	—
16α-OH-DHA	—	0.46	—
polar ketonic	—	0.06	—
nonpolar nonketonic	—	0.01	—
polar nonketonic	—	0.03	—
Time	June, 1963 ⟶		July, 1963

Subject No. 35 (31-70-95): A 26-year-old patient had a 2-year history of schizophrenia, amenorrhea and moderately severe hirsutism of the face and body.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baselines		Dexamethasone 8 mg. Q.D. 1st day	2nd day	3rd day	ACTH 25 u. I.V. in 8 h.		Surgery	Postoperative	
17-KS*	10.7	8.0	5.9	4.3	4.5	7.8	17.0	—	5.8	6.2
Porter-Silber*	6.6	6.7	4.8	2.0	0.9	7.7	20.9	—	7.6	7.5
DHA	—	0	0	—	—	—	0.17	—	—	—
16α-OH-DHA	—	0.16	0.07	—	—	—	0.32	—	—	—
polar ketonic	—	0.03	0	—	—	—	0	—	—	—
nonpolar non-ketonic	—	0.15	—	—	—	—	0.11	—	—	—
polar non-ketonic	—	0.27	—	—	—	—	0.19	—	—	—
Time	Nov., 1963 →							Jan., 1964 →		

Sclerocystic ovaries were treated by surgical wedge resection. The patient has subsequently been treated with pharmacologic doses of corticosteroids for rheumatoid arthritis, an intercurrent disease in this patient. She is amenorrheic and has more marked hirsutism.

Subject No. 38 (29-83-89): A 21-year-old student had hyper- and polymenorrhea for 6 years and noticed slowly progressive hirsutism of the upper lip but most noticeable on her arms and thighs.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baseline	2nd day of dexamethasone 2 mg. Q. D.	Cortisone 12.5 mg. Q. D.	⟶
17-KS*	23.4	11.6	10.6	10.9
Porter-Silber*	5.8	0.9	6.8	4.2
DHA	0.10			
16α-OH-DHA	0.52			
polar ketonic	0.17			
Time	Dec., 1963	Feb., 1964	March, 1964	Dec., 1964

With cortisone therapy, the patient is ovulating and menstruating regularly.

OLIGOMENORRHEIC NONHIRSUTE FEMALES

Subject No. 11 (31-76-28): A 22-year-old woman had normal menses until one and one-half years before this investigation. She had an abrupt cessation at that time. She had experienced normal sexual development.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baselines			ACTH 25 u. I. V. in 8 h.		Baseline
17-KS*	11.5	6.2	15.3	15.6	22.2	13.2
Porter-Silber*	8.9	2.2	3.5	12.5	21.5	5.5
DHA	—	0	0.07	—	—	—
16α-OH-DHA	—	0.03	0.05	—	—	—
polar ketonic	—	0.10	0.01	—	—	—
nonpolar nonketonic	—	0.03	—	—	—	—
polar nonketonic	—	0.05	—	—	—	—
Time	April, 1963	June, 1963	Oct., 1963 →			June, 1964

This patient remains amenorrheic without any therapy.

Subject No. 15 (31-02-26): A 22-year-old woman had normal menses for one year at age 12 years and had progressively less frequent menses until 18 years of age when they stopped completely.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baseline	Metyrapone 2 g. Q. D.	Baseline	ACTH 25 u. I. V. in 8 hrs.		Dexamethasone 2 mg. Q. D.	
17-KS*	16.2	26.9	10.9	14.1	20.4	5.6	1.7
Porter-Silber*	(18.0)	(28.5)	4.6	16.9	40.9	2.0	0.5
DHA	—	—	0.03	0.19	0.13	0	0.02
16α-OH-DHA	—	—	0.13	0.22	0.21	0.03	0.03
polar ketonic	—	—	0	0.17	0.03	0.03	0
nonpolar non-ketonic	—	—	0.16	0.10	0.10	0.05	0.16
polar non-ketonic	—	—	0.09	0.14	0.40	0.03	0.08
Time	Sept., 1962	Oct., 1962	Aug., 1963 →				

After cyclic estrogen therapy, the patient has resumed spontaneous periodic uterine bleeding.

Subject No. 21 (32-01-98): A 25-year-old woman experienced her pubertal skeletal and breast growth at 14 years of age but had her only menses at age 18 years. A diagnosis has not been established.

URINARY STEROIDS (mg./24 h.)

Compound(s)	Baselines		ACTH 25 u. I. V. in 8 h.	
17-KS*	11.6	10.8	11.0	21.2
Porter-Silber*	5.8	5.4	8.7	35.1
DHA	0.11	—	—	—
16α-OH-DHA	0.09	—	—	—
polar ketonic	0.25	—	—	—
nonpolar nonketonic	0.06	—	—	—
polar nonketonic	0	—	—	—
Time	July, 1963	April, 1964	April, 1964	April, 1964

The patient remains amenorrheic without any therapy.

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