STUDIES ON THE CHROMATOGRAPHIC
FRACTIONATION OF URINARY 17-KETOSTEROID,
WITH SPECIAL REFERENCE TO THE
INFLUENCE OF GONADOTROPIN

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NINE FIGURES

Urinary 17-ketosteroids have been fractionated on alumina column into eight fractions by Dingemanse, *et al.* (1946) and on a more convenient microscale by Zygmuntowicz, *et al.* (1951), Pond (1951, 1954) and Edwards, *et al.* (1953). Using the latter technic, 17-ketosteroids in the urine of young and old people, of men and women, and of castrated people and others have been studied and the relations between the secretion of gonadotropins and the function of the adrenal cortex have been discussed.

METHODS

The technic used was almost similar to the one reported by Edwards, *et al.* (1954). 1.5 g. Al_2O_3 (Japan Alumina Co. BL6, Mesh 50—60) was packed into a glass tube, 4 mm. in internal diameter and 20 cm. in height, the height of the alumina column being 105 mm.

An aliquot (Ca. 100 cc.) of a 24 hour urine was hydrolyzed and extracted by Drekter's method; i. e. adding 15 vol. % of conc. HCl. hydrolyzed for 15 minutes by heating at 80°C and then extracted with two volumes of ether twice. The combined ether extract was washed with 20 cc. of 10 % NaOH, then with 20 cc. of distilled water. The washed ether extract was evaporated to dryness and the dried residue of the crude neutral extract was obtained.

The residue was dissolved in 1 cc. of benzene and poured over an alumina column which had already been moistened with benzene.

Elution was carried out with 2 cc. portions of solvents according to the following program :

0.05 % ethanol-benzene	15 fractions
0.2 % " "	15 fractions
1 % " "	10 fractions
pure ethanol	3 fractions

Although Edwards, *et al.* eluted 30 fractions with 0.2 % E—B. from the start, the above mentioned method was used, since alumina used here was not the same in quality as that of Edwards, *et al.*

Throughout these procedures, negative pressure of 9 cm. Hg was added by water pump, and 43 fractionated eluates were obtained, dried by evaporation and the content of 17-KS present in

each eluate was measured by Dreker's modification of Zimmermann's reaction.

If the results are plotted taking the eluate numbers on the abscissa and the amount of 17-KS in $\mu\text{g.}$ on the ordinate, 8 fractions are obtained as shown in Figure 1. Each of the peaks of the curves corresponds to one or more 17-ketosteroids. Under the conditions described, a particular 17-KS is always found in the same place. (the dotted lines illustrate the places for crystalline dehydroisoandrosterone and androsterone). Eight peaks produced by the urinary extracts are recognized as follows.

- I. Non-alcoholic steroids, mainly artifacts of hydrolysis of the β -17-KS. (androst-2-en-17-one, 3-chloro-androst-5-en-17-one, androsta-3:5-dien-17-one)
 - II. i-androstanolone
 - III. dehydroisoandrosterone, isoandrosterone
 - IV. androsterone
 - V. etiocholanolone
 - VI. } 11-oxy-17-KS. { 11-keto-, & 11-hydroxy-androsterone
 - VII. } { 11-keto-, & 11-hydroxy-etiocholanolone
- } Both these fractions may include small quantities of Δ^9 -derivatives of 11-oxy-17-KS.

VIII. unidentified

According to Dingemans, *et al.*, (1952), separation of the isomers of androsterone and etiocholanolone occurs completely only when the weight of alumina is 2,500 times the weight of steroids. Since the weight of alumina used here is 1.5 g., the amount of steroid extract for fractionation should be less than 0.6 mg.

Of these eight fractions, I, II, III have been shown by Dingemans to interchange from one to the other in the course of hydrolysis. She established that fraction II became 3-chloroandrost-5-en-17-one (I) by hydrolysis with HCl at room temperature, while by hydrolysis on boiling, fraction II was converted to fraction III (DHA). Thus, as to the quantity of DHA obtained from chromatography, there is a possible inclusion of an artifact. Pond handled I, II and III together as one quantity, since these three fractions were roughly representative of the β -fraction. Ulstrom and Doeden (1954) took the sum of II and III as one quantity. In this article, these fractions are not taken into consideration, as urochromogens, which may interfere with the color developed by Zimmermann's reaction, occur in these portions, even though corrections for them are carried out.

About Urochromogens: 4 kinds of chromogens have been extracted from the urine, as shown in the upper part of Figure 1. Of these, bluish green, light yellow and brown chromogens have quite different absorption spectra from the chromogen of Zimmermann's reaction (Fig. 2), and therefore the former three do not interfere with the measurement of the latter. The pink chromogen, however, has a same absorption spectrum as the color of Zimmermann's reaction, and Fraser's correction equation cannot be applied to this chromogen. Removal of the urochromogens by Noritt (100:1) is not complete, and a loss of about 6% of steroids is found. Girard's separation appears to be incomplete in the removal of urochromogens. We have preferred the method described by Cahen & Salter, in which Zimmermann's chromogen is transferred to the chloroform layer, while urochromogens remain in the aqueous alcoholic layer.

RESULTS

Patterns in normal young people (Fig. 3)

In males, IV and V are the main fractions and the ratio of IV+V to VI+VII is over 1.0 (mean 2.5). In females two kinds of patterns are found; one pattern the same as that in the normal male (type I); and the other with very small amount of IV and V showing the ratio of only about 0.5 (type II). The latter will be discussed later.

Fig. 1. Normal pattern of the urinary 17-KS.

Fig. 2. Absorption spectra of four kinds of chromogens.

Fractions IV and V, androsterone and etiocholanolone, derive from testosterone in the male, but in the female, especially those showing type I, it is most reasonable to consider that fractions IV and V originate from the adrenal cortex.

Pattern in normal elderly people (Fig. 4)

Not only aged males, but also aged females show the same pattern as the young male, IV and V being the main fractions. No aged female shows the type II pattern like some young females.

If the fractions IV and V in the young male are of testicular origin, it is very interesting that the ratio of IV+V to VI+VII remains almost constant even in the aged in whom the secretion of testosterone has already decreased.

Fig. 3. Patterns in normal young people.

Fig. 4. Pattern in normal elderly people.

Pattern in the castrated (Fig. 5)

From the findings in young females and in the aged, it may be easily understood that fractions IV and V originate not only from the testis, but also from the adrenal cortex. The chromatographic separation of the urinary 17-KS in 4 castrates, 3 men and one woman, were undertaken. As shown in Figure 5, fractions IV and V are rather large in these three cases as compared with those in aged males and the ratio is 2.83 (mean of three castrated men). It is clear that the proportion of androsterone+etiocholanolone to 11-oxy-17-KS does not decrease even in the absence of the testes.

The pattern of an ovariectomized woman is the same as type I of the young female, shown in the lowest column of Figure 3.

Patterns in the person who lacks in gonadotropin (Fig. 6)

However, in a man with pituitary hypogonadism, 28 years old, as well as a male infant, 5 years old, a pattern similar to type II of the young female is found, and the ratio of IV+V to VI+VII is about 0.5. In these two cases there is almost no testicular secretion, a situation similar to that in the castrated. But in these cases the secretion of gonadotropin is very low, while it is very high in castrated.

It appears definite that testosterone can be a precursor of androsterone and etiocholanolone but this is not always the case. Even in the castrated, the ratio of IV+V to VI+VII never decreases if the secretion of gonadotropin is sufficient. Similarly IV+V in the aged does not decrease in amount, since the secretion of gonadotropin is stimulated as the result of the decrease in testicular secretion. On

Fig. 5. Pattern in the castrated.

the other hand, when the secretion of gonadotropin is small, the output of IV+V, both from testes and presumably from adrenal is very low.

Influences of gonadotropin administration on the fractions of 17-ketosteroids. (Fig. 7)

Gonadotropin was administered experimentally to a 20-year-old woman complaining of amenorrhoea. Antex Leo (pregnant mare serum gonadotropin) was administered subcutaneously, 1500 I. U. daily for five days, and the administration of physex Leo (chorionic gonadotropin) 1500 I. U. daily for the following six days longer.

The total 17-KS, 4.4 mg. on the day before treatment, increased to 5.9 mg. on the 4th and 9th day of injection but it is not clear whether this was a true increase or only a daily variation, since the excretion on the 11th day returned to 4.1 mg. No remarkable change of the total 17-KS output was therefore observed during the experiment.

However the ratio of IV+V to VI+VII increased gradually and significantly, 1.92 (before), 2.66 on the 5th day, 2.72 on the 7th day and 5.02 on the 11th day. Moreover the increased amount of the fractions IV+V roughly corresponded with the decreased amount of the fractions VI+VII, and hence the amount of the total 17-KS remained almost constant.

Fig. 6. Pattern in the person who lacks in gonadotropin.

Fig. 7. Influence of gonadotropin administration on the fractions of 17-KS.

Postulated Relationships between gonadotropin and 17-ketosteroid in various conditions.

From the results described above, the relationship between gonadotropin and 17-KS fractions in various conditions can be summarized as in Figure 8.

In the male; if gonadotropin secretion is poor as in infants or hypogonadotropic hypogonadism, fractions IV and V are very small, as they are not produced in quantity either from the testes or from the adrenal cortex. In young males they are derived abundantly from the very active testes and, in aged males when testicular secretion decreases gradually, the augmented gonadotropin stimulates the adrenal cortex to cause an increase in the amount of IV and V, and a similar chromatographic pattern can be observed as in the young male. In castrated men with remarkably increased gonadotropin, these two fractions, derived exclusively from the adrenal cortex, never does decrease.

In the female; in infants and hypogonadotropic hypogonadism there exist the same relations as in the male. In the young female two types have been found as already described. Type I seems to be found in the period of high gonadotropin level and type II in the other period of the menstrual cycle. In one woman aged 30, the ratio of IV+V to VI+VII was 0.91 on the 5th day of menstruation, while the ratio became 2.83 on the 15th day. Such a change of the ratio in a particular case could never be found in males. In females, therefore, there must be various values of the ratio, according to the cyclic variation of the secretion of gonadotropin.

Fig. 4. Relationships between gonadotropin and fractions of 17-KS in various conditions.

Fig. 9. Steroidogenetic processes, wherein C₁₁-hyd.oxylation does not take place.

DISCUSSION

It appears to be sure that gonadotropin increases the ratio of IV+V to VI+VII by stimulating the adrenal cortex, but the mechanism of this action is not yet at all clear. We would like to present the following hypothesis based on our present knowledge.

Some of the 17-ketosteroids are excreted in the urine as metabolites of some

of the corticosteroids (except testicular origin), which have been synthesized in the adrenal gland from cholesterol or acetate. Since gonadotropin influences the ratio of IV+V to VI+VII, it must act on some phase or phases of this process. It seems more reasonable that this action is on the process of biosynthesis of corticosteroids in the adrenal gland, rather than on the pathways of degradation of corticosteroids outside of the adrenal gland.

According to the excellent work of Pincus, Hechter and others (1954), corticosteroidogenesis may take place in the following manner.

Thus far, then, there have been found four kinds of specific enzymes in the adrenal cortex, a dehydrogenase of Ring A, and hydroxylases at C₁₇, C₂₁ and C₁₁. An assumption has been made, that gonadotropin stimulates the former three actions, causing a relative fall of the activity of hydroxylation at C₁₁.

Figure 9 indicates, for simplicity, an extreme case of corticosteroidogenesis, wherein C₁₁-hydroxylation does not take place at all. Even if the biosynthesis proceeds as in the top line, 17-OH-desoxycorticosterone (Reichstein's S) leaves the adrenal gland, since it is now unable to become compound F. And it is excreted in the urine in the form of androsterone and etiocholanolone, probably via androstenedione. Of course, 17-OH-progesterone itself may leave the adrenal gland and similarly appear in the urine as androsterone and etiocholanone.

It may be the case that progesterone itself comes out into the blood stream, but it will appear in the urine as pregnandiol, never as 17-KS, because it does not have a hydroxyl group at C₁₇.

On the second line, where Δ^5 -pregnenolone is first subjected to hydroxylation at C₁₇, Δ^5 -pregnenediol-3, 17, and then Δ^5 -pregnenetriol-3, 17, 21 is produced, and dehydroisoandrosterone (DHA) may appear finally in the urine. On the other hand, if Δ^5 -pregnenediol-3, 17 or Δ^5 -pregnenetriol-3, 17, 21 is subjected to oxydation at ring A, each may become 17-OH-progesterone or 17-OH-desoxycosterone respectively and may be excreted finally in the urine as androsterone and etiocholanolone.

The third line indicates the possibility in which Δ^5 -pregnenolone is first subjected to hydroxylation at C₂₁. Here one would have Δ^5 -pregnenediol-3, 21 which will be excreted in the urine as Δ^5 -pregnenetriol-3, 20, 21. Otherwise there may be some other pathways of hydroxylation at C₁₇ and oxydation of ring A. (the second and the first line) and thus there may occur possible yielding DHA, androsterone and etiocholanone.

The fourth line (lowest) indicates that hydroxylation occurred first at C₁₁, but this process is inhibited by gonadotropin according to an assumption mentioned above.

In the column on the extreme right, some types of compounds excreted in the

urine are indicated. Of them, pregnanediol and Δ^5 -pregnenetriol-3, 20, 21, are not 17-ketosteroid and these two are therefore out of our consideration.

The total quantities of compounds produced are decided by the amount of the starting material, Δ^5 -pregnenolone, which in turn is influenced by ACTH. However, since the quantity of ACTH can be considered to be unchanged, the amount of Δ^5 -pregnenolone produced is also settled and hence the total amounts of 17-KS excreted in the urine can be considered to be virtually fixed.

Now one may postulate that gonadotropin acts to alter the proportions of the constituents of the total 17-KS, i.e., increase IV+V and decrease VI+VII, although it may not influence the amount of total 17-KS. Neither increase nor decrease of DHA (fraction III) may occur with gonadotropin in this scheme.

The assumption has been made that gonadotropin accelerates the actions of three specific enzymes of the four of the adrenal cortex, but it has been recently postulated that no tropic hormone such as ACTH is necessary for the activities of those enzymes. With these considerations perhaps gonadotropin stimulates the synthesis of those three enzymes, bringing about a relative shortage of the enzyme concerning C_{11} -hydroxylation.

Similar conditions have been found about ACTH by Kass, Hechter, *et al.* (1954). Adrenal venous blood collected from non-treated rabbits and from rabbits which had received ACTH for a 3 hour period, showed the presence of only compound B. When, however, the rabbits were treated with ACTH for 21 to 28 days, the predominant corticosteroid in the adrenal venous sample was compound F, although compound B was also present. When the rabbits were treated with ACTH for seven days, the ratio of F to B was 1:2.

This finding supports the view that chronic ACTH treatment increased the activity of the C_{17} -hydroxylating enzyme from the minimal values present in the normal rabbits to significant levels. Thus ACTH, in addition to its rapid action on corticosteroidogenesis, is also concerned with the synthesis of a specific enzyme of the adrenal cortex.

Dobriner, *et al.* (1954). found an increase in 11-oxy-17-KS and a decrease in androsterone and etiocholanolone with the daily injection of 100 mg. of ACTH for four days. Samuels (1953) has stated that gonadotropin indirectly stimulates the oxydation of ring A of steroids present in the testes by promoting the synthesis of this protein enzyme.

Thus, by the assumption that gonadotropin is concerned with the synthesis some specific enzymes, our above described experiment in which the ratio of IV+V to VI+VII gradually increased to the maximal value on the 11th last day, may be readily explicable. However, the kind of gonadotropin which plays the greater role in this action, cannot be established from our experiment. According to the finding of Dobriner and Lieberman (1952) that androsterone in the urine became nil in the last trimester of pregnancy, and from the knowledge that FSH is the chief gonadotropin which increases in the aged; FSH, rather than LH, may be the important one.

SUMMARY

Urinary 17-Ketosteroids have been separated into 8 fractions by the adsorption chromatography. Of these 8 fractions, dehydroisoandrosterone (III) and 11-oxy-17-KS (VI and VII) derive exclusively from adrenal cortex, but androsterone (IV) and etiocholanolone (V) have been thought to be of testicular origin.

The latter two compounds (IV & V), however, are not always testicular. If gonadotropin secretion is sufficient, these two fractions exist in the same ratio in the urine of the female as well as of the aged, and even in the urine of the castrated. In men with decreased gonadotropin, infant or hypogonadotropic hypogonadism, the amount of these two fractions in the urine are very small and the chromatographic pattern differs from that of the normal male, IV+V being the major fraction. In the normal female, two kinds of patterns have been found; the one is the same with that of the normal, the other is the same with that of infant. The former seems to be found in the period of high gonadotropin level and the latter in the other period of the menstrual cycle. In the aged female only the former type is found, because she is always in the state of high gonadotropin level. Moreover, in a 20-year-old female, these two fractions (VI & V) could be increased by the administration of gonadotropin without any change in the amount of total 17-KS.

From the above mentioned, it can be postulated that gonadotropin acts to alter the proportions of the constituents of the total 17-KS, i. e. increase IV+V and decrease VI+VII, by stimulating the adrenal cortex. The mechanism of this action of gonadotropin is discussed.

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