CXXVI. ISOLATION OF $\Delta^{3:5}$ -ANDROSTADIENE-17-ONE FROM THE URINE OF A MAN WITH A MALIGNANT TUMOUR OF THE ADRENAL CORTEX

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HYPERPLASIA or neoplasia of the adrenal gland, as is well known, may cause changes in the accessory sexual attributes of the patient. The changes differ in the two sexes. In women, in whom these adrenal disorders are more frequent than in men, virilism is commonly produced: coarse hair grows on the face and other parts of the body where its distribution comes to resemble that of the male, the breasts and genitalia atrophy, the menses no longer appear and the voice may become masculine in tone. In men, on the other hand, feminism may be caused by these adrenal disturbances: the hair of the face becomes scanty, the breasts swell and may even produce milk, the testes and genitalia atrophy and impotence ensues. Hypothetically the changes thus produced might be attributed to an incorrect proportion between the male and female hormones. An increased output of male hormone has been found in the urine of females suffering from adrenal virilism. In the case of adrenal feminism in a male about to be described there was an increased output of oestrogenic hormone. This excessive production of oestrogenic material had not hitherto been observed in a male with an adrenal tumour, though Frank [1934] has recorded two cases of adrenal tumour in women accompanied by an excessive output of oestrogenic hormone. The primary object of the work now described was the isolation and identification of the oestrogenic hormone. The following are brief clinical notes. The patient, a man of 34 years, was under the care of Dr Levy Simpson. An adrenal tumour was present and caused signs of feminism: the breasts were enlarged, the testes reduced in size and there was impotence. The adrenal tumour was removed by Mr C. A. Joll on 22 July 1934, and weighed 660 g. A specimen of urine passed on the morning of that day and submitted to qualitative tests showed an absence of the prolan reaction and the presence of oestrogenic hormone in a concentration of at least 500 mouse units per litre. At the operation the tumour was found to have invaded the adrenal vein; recurrence of the growth therefore seemed likely. After removal of the tumour the excessive output of oestrogen ceased for a while. In February 1936, however, it became clear that metastatic growths were present, and from this date until the patient's death, 3 months later, his urine was collected for analysis, some 30 l. in all being obtained.

Isolation of new "hormones"

After hydrolysis of the urine with hydrochloric acid, an assay of the oestrogenic hormone gave a value of approximately 3000 I.U. per l. We have not ourselves estimated the male hormone content, but a sample of urine from this

case taken shortly before the beginning of the collection of our 30 l. was assayed by Dr de Fremery, who found the comb-growth promoting activity to be between 50 and 100 I.U. Comparison of these values with data given in the literature show that the male hormone content is not excessively high, but the oestrogen content is approximately 20 times that found in normal male urine. For instance, Gallagher *et al.* [1935] quote values ranging from 18 to 84 I.U. of male hormone, and 80–110 I.U. oestrogenic hormone [see also Borchardt *et al.* 1934; Callow, 1936, 1]. The figures quoted above perhaps convey a somewhat misleading impression of the relative amounts of male and oestrogenic hormones present in the urine, and it should be remembered that whereas 100 I.U. of male hormone activity correspond with 10 mg. of androsterone, 2500 I.U. of oestrogenic activity correspond with only 0.25 mg. of oestrone. Thus, assuming the oestrogenic activity to be due entirely to oestrone, the total amount present in the 30 l. under examination would not exceed 10 mg., from which it is clear that isolation and identification would not be a simple task.

As the result of our fractionation of the 30 l. of urine we have isolated and identified 0.75 g. of *p*-cresol, and approximately 0.4 g. of an unsaturated ketone, $\Delta^{3:5}$ -androstadiene-17-one (III), which has no demonstrable oestrogenic activity but has weak comb-growth promoting activity, sufficient to account for the male hormone activity found by Dr de Fremery in the urine from this case. We have also isolated (in the form of derivatives) small amounts of two other ketones, neither of which has marked oestrogenic activity. The analytical figures suggested that these ketones, derived respectively from the "phenolic" and the "neutral" fractions of the urine are represented by the formulae $C_{19}H_{28}O_3$ and $C_{21}H_{32}O_3$.¹ These formulae are not established with certainty for the amounts of material isolated were too small for us to be sure that complete purification had been achieved. On the assumption that these ketones are related to the sterols, the C_{19} compound would seem to be an unsaturated ketone of the androstane group, and the fact that it was separated from the "phenolic" fraction suggests the presence of a β -diketone grouping. The C_{21} compound may likewise be assigned to the pregnane group, and could be represented by a structure such as (I) which is, however, purely hypothetical.

We have not succeeded in isolating the oestrogenic principle of the urine, but we were able to concentrate practically the whole of the oestrogenic material in a mixture of semicarbazones separated from the "phenolic" fraction, and in our view the properties and solubility of this mixture are consistent with the hypothesis that the oestrogenic substance is essentially oestrone. The isolation of the pure semicarbazone was prevented by the presence in the fraction in question of substances yielding more sparingly soluble semicarbazones.

¹ This compound may be related to the substance, to which is attributed the formula $C_{21}H_{36}O_3$, recently isolated by Marrian and Butler from the urine in a case of virilism. In reporting this work Broster & Vines [1937] suggest, apparently in ignorance of the work of Callow [1936, 2], that this new compound is specific to, and characteristic of, virilism.

The three ketones which we have isolated have not hitherto been encountered among the constituents of urine, and there is little doubt that they are abnormal constituents due to the pathological state of the patient, although it is possible that they are present in normal urine, but in such low concentration that their presence has not been recognized. Furthermore, it is evident that the presence of these substances in the urine is not so much due to the fact that the patient was suffering from cancer, as to excessive growth of adrenal cortical tissue, which is known to produce a variety of substances of similar chemical nature to those which we have isolated; among them is the cortical hormone, corticosterone [cf. Mason *et al.* 1936; Reichstein, 1936, 1; de Fremery *et al.* 1937; Wintersteiner & Pfiffner, 1935; 1936]. That the tissue involved in the tumour was the adrenal cortex was suggested by histological examination of the excised tumour, which showed considerable resemblance to normal cortical tissue.

$\Delta^{3:5}$ -Androstadiene-17-one is so readily isolated by the technique which we have adopted that it is inconceivable that it should not have been isolated already if it were present in normal urine in appreciable amount. As a control experiment, we have made a similar extraction of a pooled sample of 30 l. of urine from male patients with cancer in other tissues, mainly in the tongue and throat, and we have not obtained any indication of the presence of this ketone.

It will be seen from the considerations advanced in the next section that there is a close relationship between the unsaturated ketone which was so abundantly excreted by the male patient with an adrenal tumour, and dehydroandrosterone, of which it is a product of dehydration. Callow [1936, 2] isolated abnormally large amounts of dehydroandrosterone from the urine of a female patient, with symptoms of virilism, suffering from an adrenal tumour, and it is a reasonable inference that the over-production of male hormone was responsible for the abnormal sexual characteristics. In the case which we have examined it is a little more difficult to account for the symptoms of feminism, with atrophy of the testes, for increased output of oestrogenic hormone seems to be compensated by the large excretion of a ketone which has male hormone potency roughly equal to that of dehydroandrosterone. However, Reichstein showed that one of the ketones which he isolated from the adrenal cortex had comb-growth activity, but was without influence on the seminal vesicles of castrated rats, so that comb-growth activity does not necessarily mean that the compound in question would function as a male hormone in a human subject. It will be of interest to study the influence of our new ketone on the sex organs of castrated male rats.

Identification and artificial preparation of $\Delta^{3:5}$ -androstadiene-17-one

This unsaturated ketone was characterized by its *oxime* and its *semicarbazone*, and the analytical figures of the ketone and its two derivatives were all in satisfactory agreement with the formula $C_{19}H_{26}O$. The presence of two ethylenic linkages was demonstrated by measurement of the hydrogen absorption in catalytic microhydrogenation experiments kindly carried out by Mr H. Jackson of the University of Manchester.¹ The product of hydrogenation with a palladium catalyst was a saturated ketone, $C_{19}H_{30}O$, a result strongly indicating a relationship with androstane, $C_{19}H_{32}$, the parent hydrocarbon of the male hormone group, or its stereoisomeride aetiocholan. Such a relationship was proved by Kishner-Wolff reduction of the saturated ketone to androstane, and the 17-position of the carbonyl group was established by the demonstration that the saturated ketone was identical with the known 17-androstanone. It remained

¹ We are much indebted to Prof. I. M. Heilbron for his courtesy in affording the facilities of his laboratory for these determinations.

then to determine the positions occupied by the two ethylenic linkages of the original unsaturated ketone. Measurement of the ultraviolet absorption spectrum, details of which are given in the succeeding section, eventually suggested the positions 3:4 and 5:6 for these, and confirmation of this was afforded by the preparation of the same unsaturated ketone by dehydration of dehydroandrosterone (II). The preparation of the ketone, and its stepwise conversion into androstane may thus be represented as follows:

During the course of the experiments which led to this conclusion consideration was given to the possibility that the compound isolated from the urine was an $\alpha\beta$ -unsaturated ketone containing the grouping shown in formula (VI). It would be expected from analogy that such a compound would be reduced to 3-aetiocholestanone (VII) and then aetiocholane (i.e. hydrogenation would lead to *cis*-linkages between rings I and II).

Hence we were interested in the preparation of 3-aetiocholestanone and attempted to obtain it by Wolff-Kishner reduction of dehydroandrosterone, followed by oxidation of the dibromide of the unsaturated alcohol which we expected to obtain. However, when the semicarbazone of dehydroandrosterone was heated with sodium ethoxide not only was the carbonyl group reduced but also the double bond, to the extent of about 90%. Hydrogenation of a double bond at the 5:6-position of the sterol molecule invariably leads to *trans*-linkages between rings I and II, so that there can be little doubt that the saturated ketone which we isolated is 3-androstanone (VIII), a compound not hitherto described.

The compounds related to the sterols which have been isolated from urine have always contained hydroxyl groups and some, at any rate, are present in the urine in combination with a glycuronic acid. Thus the unsaturated ketone which we have isolated from the pathological case under examination represents an unusual type of urinary constituent, and it seems likely that the ketone was not present as such in the original urine. We offer no suggestion as to the

probable nature of the precursor, except to express the view that this is unlikely to be dehydroandrosterone, which is not dehydrated by the acid hydrolysis to which the urine was subjected before extraction. At first sight it seemed that such dehydration might occur if *epidehydroandrosterone* were the precursor, for Marker *et al.* [1936] have shown that *epicholesterol* is very sensitive to dilute mineral acid, by which it is readily dehydrated to cholesterolene. *epidehydroandrosterone* has recently been prepared by Ruzicka & Goldberg [1936], and in order to test this hypothesis we treated *epidehydroandrosterone*¹ with alcoholic hydrochloric acid under conditions which suffice for dehydration of *epicholesterol*. However, dehydration to $\Delta^{3:5}$ -androstadienone did not occur.

Ultraviolet absorption spectra

The principal abnormal constituent which was isolated from the urine contained a carbonyl group and two ethylenic linkages, and it was therefore evident that its absorption spectrum would afford information as to whether these unsaturated centres were conjugated. The spectrum of the ketone (Fig. 1) shows intense absorption at 2345 Å. ($\log \epsilon = 4.258$) with a broad region of weak absorption at 2600–3200 Å. ($\log \epsilon = 2.044$ at 2770 Å.). This is very similar to the absorption shown by a number of $\alpha\beta$ -unsaturated ketones of the sterol group (e.g. Δ^1 - and Δ^4 -cholestenone, progesterone, testosterone, Δ^4 -androstene-3:17-dione). The presence of a triply conjugated system was excluded, as such a system would be expected to show more intense absorption with the maximum displaced to the 2800 Å. region, as, for example, in oxycholesterilene [Rosenheim & King, 1934] and crotylideneacetone [Smakula, 1934]. There was, however, the alternative possibility that the two ethylenic bonds were conjugated with one another, but separated from the carbonyl group. At first sight this seemed to be untenable, as although simple conjugated dienes such as butadiene, dimethylbutadiene, isoprene and *cyclopentadiene* show similar intense absorption in the region 2200–2400 Å. (International Critical Tables, 1929, 5, 363), if the two double bonds form part of a 6-membered ring the absorption is profoundly modified and becomes benzenoid in character. This is true of *cyclohexadiene* [Allsopp, 1934], ergosterol [Heilbron *et al.* 1928], ergostatriene and $\Delta^{5:7}$ -cholestadiene [Dimroth & Trautmann, 1936].²

The kind of conjugated diene typified by cholesterolene, in which the double bonds are in adjacent 6-membered rings, was found, on detailed examination, to give the same type of absorption as is shown by our ketone and by the simple conjugated dienes such as butadiene. Hence the spectroscopic evidence showed that the unsaturated ketone contained either an $\alpha\beta$ -unsaturated ketone grouping, or two conjugated ethylenic bonds in adjacent rings with the carbonyl group in some other part of the molecule.³

Cholesterolene shows a broad absorption band, with a maximum at 2350 Å. ($\log \epsilon = 4.302$) and slight absorption at 2700–2800 Å. Müller & Page [1933] found that the long wave-length absorption decreases on purification, and

¹ Prof. L. Ruzicka very generously gave us a specimen of this difficultly accessible compound.

² Note added in proof. The compound denoted as $\Delta^{2:4}$ -cholestadiene by Schoenheimer & Evans [1936] has recently been shown to have another structure by Stavely & Bergmann (*J. org. Chem.* 1937, 1, 575) who have also prepared the true $\Delta^{2:4}$ -cholestadiene. They state that this has an absorption maximum at 2600 Å. a result consistent with the view expressed above concerning the type of absorption associated with two conjugated double bonds in a six-membered ring.

³ In the preliminary report of this work Burrows *et al.* [1936] we suggested that the ketone isolated from urine was an $\alpha\beta$ -unsaturated ketone. In arriving at this conclusion we had been misled by the incomplete absorption curves published for cholesterolene.

Rosenheim & King [1934] concluded from this and from the differences between their absorption curve for cholesterolene and that published by Heilbron *et al.* [1928] that the absorption in this region is due to impurity. Our own experiments support this view. A sample of cholesterolene prepared by the method of Müller & Page [1933] showed much less of this long wave-length absorption (see Fig. 2) than a specimen prepared by the method of Mauthner & Suida [1896]. Moreover, a sample of $\Delta^{3:5}$ -androstadiene-17-one prepared by dehydration of dehydroandrosterone showed twice as much absorption in this region as the ketone isolated from the urine, but the main intense absorption bands were identical for the two samples (Fig. 1).

Fig. 1.

Fig. 2.

It seems probable that the impurities in cholesterolene and $\Delta^{3:5}$ -androstadiene-17-one responsible for the absorption at 2700–2800 Å. are isomeric dienes in which the double bonds have migrated so that they are both in one ring, thus giving rise to the complex type of absorption characteristic of ergosterol, which has its maxima in this region, although in the case of androstadienone this absorption is doubtless partly due to the isolated carbonyl group in the molecule.

EXPERIMENTAL

The material used in this investigation was approximately 30 l. of urine, collected during the 3 months immediately preceding the death of the patient. The urine was submitted to preliminary hydrolysis by boiling under reflux for 2 hr., after addition of 100 ml. of conc. HCl per l. After this treatment the urine was extracted with ether for 24 hr. in a continuous extractor, in portions of 1500 ml. The combined ethereal extract was washed free from acid with aqueous NaHCO_3 , and evaporated. In order to separate "phenolic" substances the viscous reddish brown oil was dissolved in *n*-butyl alcohol (50 ml.), and the solution treated with light petroleum (150 ml.) and then shaken 20 times with 0.25 *N* NaOH (portions of 50 ml.). The alkaline extracts were at first dark brown and tarry, and later pale yellow. The combined alkaline extract was

acidified with HCl and extracted with ether for 24 hr. After washing the extract with aqueous NaHCO_3 and with water, the ether was evaporated. The residue was dried in a vacuum over conc. H_2SO_4 for 10 days, and gave 9.4 g. of a brown syrup ("phenolic" fraction). The butyl alcohol and light petroleum were distilled off from the solution, after washing with alkali, and gave about 5 g. of a clear red gum (neutral fraction).

Treatment of neutral fraction

A solution of the gum in alcohol (50 ml.) was treated with an aqueous solution of semicarbazide hydrochloride (4 g.) and sodium acetate (4 g.) and the whole boiled for 4 hr. A crystalline solid began to separate after a few minutes and gradually increased in quantity. After cooling in the ice-chest the semicarbazone was collected and washed with water and with ether. The yield was 1.4 g. of a pink microcrystalline powder, m.p. 262–265°. This product (1.15 g.) was extracted 6 times with boiling alcohol (boiled for 1 hr. with portions of 60 ml.). The insoluble residue (635 mg.) had m.p. 264–266°. The alcoholic extract was concentrated to one-third of its volume, and cooled, when a solid crystallized (135 mg.), m.p. 257–259°. Both this and the insoluble residue were shown to consist of the semicarbazone of $\Delta^{3:5}$ -androstadiene-17-one, the isolation of which is described below. The alcoholic mother-liquors were evaporated to dryness and the material thus obtained was combined with a further quantity of semicarbazone obtained by dilution of the aqueous alcoholic liquors of the original preparation. This mixture of more soluble semicarbazones (550 mg.) was hydrolysed by 30 min. boiling with a mixture of alcohol (75 ml.), conc. H_2SO_4 (10 ml.), and water (15 ml.). The clear solution was diluted with water, extracted with ether, and the extract washed, dried (sodium sulphate), and the ether removed. The residue was distilled from a bath at 120°, at about 0.001 mm., and the liquid distillate (200 mg.) was heated in aqueous alcoholic solution for 4 hr. with hydroxylamine hydrochloride (1 g.) and sodium acetate (1 g.). The solution was poured into water, and next day the crystalline precipitate was collected and recrystallized from methyl alcohol, when it had m.p. 168–178°. This mixture of oximes was sublimed at 150° in a high vacuum and recrystallized twice from methyl alcohol. The resulting *oxime* (7.5 mg.) formed colourless needles, m.p. 200–202°, after sintering at 195°, and gave an orange-red colour with conc. H_2SO_4 . There was insufficient material for further purification. Analysis suggested that this was an oxime of a ketone $\text{C}_{21}\text{H}_{32}\text{O}_3$ (I?). (Found: C, 73.7; H, 9.6%. $\text{C}_{21}\text{H}_{33}\text{O}_3\text{N}$ requires C, 72.6; H, 9.6%.) The liquors from the final two crystallizations of the oxime were concentrated and gave 3.5 mg. of material, m.p. 187–192°, after sintering. This was hydrolysed with aqueous alcoholic H_2SO_4 and tested for oestrogenic action.

Isolation of $\Delta^{3:5}$ -androstadiene-17-one (III)

The sparingly soluble semicarbazone (635 mg.) obtained from the neutral fraction as described in the preceding section was hydrolysed by 30 min. boiling with a mixture of alcohol (75 ml.), conc. H_2SO_4 (10 ml.) and water (15 ml.). The product was isolated in the usual way and distilled at about 0.001 mm. from an air bath at 100°. The colourless distillate slowly crystallized and was recrystallized from aqueous methyl alcohol. The resulting $\Delta^{3:5}$ -androstadiene-17-one (0.25 g.) formed colourless leaflets, m.p. 88–89°, not depressed by admixture with a specimen prepared by dehydration of dehydroandrosterone (see below). (Found: C, 84.3, 84.2; H, 9.9, 9.8%. $\text{C}_{19}\text{H}_{26}\text{O}$ requires C, 84.4; H, 9.7%.) This ketone had $[\alpha]_D^{20} = -30.4^\circ$ (*c* in ethyl alcohol = 2.04). The *oxime*, prepared in aqueous

alcoholic solution, crystallized from methyl alcohol, in which it is easily soluble, in colourless needles which had m.p. 164–170°, after sintering at 160°. A sharp-melting product could not be obtained; presumably this was a mixture of *syn* and *anti* oximes. (Found: C, 79.9, 79.9; H, 9.6, 9.6; N, 5.75%. $C_{19}H_{27}ON$ requires C, 79.95; H, 9.55; N, 4.9%.) The *semicarbazone*, prepared from the pure ketone, was very sparingly soluble in alcohol. It crystallized from dioxan in microscopic silky needles, m.p. 291–292°. The m.p. is dependent on the rate of heating. This value was obtained by introducing the specimen into a bath previously heated to 255° and heating at such a rate that the determination was complete in 2 min. With slower heating values *ca.* 275–280° are obtained. (Found: C, 72.8; H, 9.0; N, 12.7%. $C_{20}H_{29}ON_3$ requires C, 73.3; H, 8.9; N, 12.8%.)

When the pure ketone was isolated it was shown by qualitative tests (orange-brown colour with H_2SO_4 ; decoloration of pyridine sulphate dibromide; reduction of boiling permanganate solution) to be unsaturated. The presence of two ethylenic linkages was demonstrated by microhydrogenation: (a) with a Pd-BaSO₄ catalyst the hydrogen absorption was equivalent to 2.15 mol.; (b) with a PtO₂ catalyst, which would also reduce the carbonyl group, the hydrogen absorption was equivalent to 2.82 mol.

17-Androstanone (IV)

An alcoholic solution of the unsaturated ketone (50 mg.) was shaken with palladium-black (25 mg.) and hydrogen for 3½ hr. The product crystallized from aqueous methyl alcohol in colourless leaflets, m.p. 118–120°, and gave no colour with conc. H_2SO_4 . Its m.p. was not depressed by admixture with an authentic specimen of 17-androstanone, very kindly supplied by Prof. A. Butenandt. (Found: C, 82.7; H, 11.0%. Calc. for $C_{19}H_{30}O$, C, 83.1; H, 11.0%.) The *semicarbazone* crystallized from dioxan as a microcrystalline powder, m.p. 287–288°, in a bath preheated to 250°. (Found: C, 72.1; H, 9.9%. Calc. for $C_{20}H_{33}ON_3$, C, 72.4; H, 10.0%.) The m.p. of this *semicarbazone* has been variously given as 263–266° [Butenandt & Dannenbaum, 1934], 270° [Ruzicka *et al.* 1935] and 275° [Fernholz & Chakravorty, 1935]. The identification of this saturated ketone was completed by reduction to the parent hydrocarbon, androstane (V), for which purpose the *semicarbazone* (100 mg.) was heated with sodium ethoxide (from 0.2 g. of sodium and 3–4 ml. of alcohol) at 180° for 18 hr. The product was extracted with ether, and distilled at 0.02 mm. The androstane distilled at 85–90° (bath temperature) as a colourless gum which was dissolved in acetone and the solution strongly cooled. The resulting crystals had m.p. 49°, alone or mixed with an authentic specimen of androstane prepared by Clemmensen reduction of 3:17-androstanedione [compare Butenandt & Tscherning, 1934]. In the above distillation of the Wolff-Kishner reduction products a higher fraction sublimed at 120–140°. This was recrystallized from hexane and gave about 3 mg. of colourless leaflets, m.p. 156–158°. This was clearly a 17-hydroxy-androstane formed by incomplete reduction of the ketone; a compound of similar m.p., which could be oxidized to 17-androstanone, was obtained by Reichstein [1936, 2] as a by-product in the Clemmensen reduction of androstanedione.

Examination of “phenolic” fraction

Separation of ketonic constituents. A solution of the crude brown syrup in alcohol (40 ml.) and glacial acetic acid (5 ml.) was heated on the water-bath for 1 hr. with trimethylaminoacetohydrazide chloride (5 g.) [Girard & Sandulesco, 1936]. After cooling and diluting with water, the ice-cold solution was

treated with 3 g. of NaOH (sufficient to neutralize 9/10 of the acetic acid), and then extracted 3 times with ether. The ether extract was dried and the ether removed ("non-ketonic phenolic" fraction). The aqueous solution was treated with dil. H_2SO_4 (containing 10 ml. of conc. acid) so that the total volume was about 500 ml. After an hour at room temperature the whole was extracted with ether, the ethereal extract dried (sodium sulphate), the ether removed on the water-bath and the residue (about 0.5 g.) heated with semicarbazide in aqueous alcoholic solution for $2\frac{1}{2}$ hr. The mixture of semicarbazones thus formed was separated by means of alcohol into three main fractions. Fraction A consisted of an insoluble residue which remained after repeated extraction with boiling alcohol. This had m.p. about $275\text{--}280^\circ$, and there was insufficient for further examination. Fraction B crystallized when the alcoholic extracts were concentrated. This was recrystallized from alcohol (charcoal) and then formed fern-like clusters of small colourless needles, m.p. $269\text{--}270^\circ$, which depressed the m.p. of oestrone semicarbazone. The yield was about 10 mg. Analysis suggested the semicarbazone of a ketone $\text{C}_{19}\text{H}_{28}\text{O}_3$. (Found: C, 66.9; H, 9.1; N, 11.3%. $\text{C}_{20}\text{H}_{31}\text{O}_3\text{N}_3$ requires C, 66.4; H, 8.65; N, 11.6%.) The hydrogen figure is considerably too high for the compound to contain an aromatic ring in a sterol type of molecule, and the appearance of this compound in the "phenolic" fraction must be ascribed to the presence of some other acidic group (e.g. a β -diketone) or to mere physical solution in the alkaline phenoxides. Fraction C was a mixture of semicarbazones of indefinite m.p. obtained by diluting with water the alcoholic liquors of fractions A and B. Fraction C contained the bulk of the oestrogenic material. Moreover, oestrone, if present, would have appeared in this fraction.

Isolation of p-cresol. The "non-ketonic phenolic" fraction, i.e. that portion of the phenolic fraction which did not react with Girard's reagent, was distilled at 25 mm. At 150° (bath temperature) 0.75 g. of a colourless evil-smelling liquid distilled. This was completely soluble in dilute alkali and was shown to be essentially *p*-cresol. Its benzoate had m.p. $68\text{--}69^\circ$, alone or mixed with an authentic specimen. For further characterization two new derivatives were prepared. The 3:5-dinitrobenzoate crystallized from alcohol in colourless flat needles, m.p. 180° . (Found: C, 55.6; H, 3.3; N, 9.3%. $\text{C}_{14}\text{H}_{10}\text{O}_6\text{N}_2$ requires C, 55.6; H, 3.3; N, 9.5%.) The m.p. was not depressed by a specimen, m.p. 186° , prepared from pure *p*-cresol. The *p*-phenylbenzoate crystallized from alcohol in colourless leaflets, m.p. $124\text{--}125^\circ$. (Found: C, 82.9; H, 5.5%. $\text{C}_{20}\text{H}_{16}\text{O}_2$ requires C, 83.3; H, 5.6%.) A specimen prepared from pure *p*-cresol had m.p. 126° .

$\Delta^{3:5}$ -Androstadiene-17-one from dehydroandrosterone (II)

A mixture of dehydroandrosterone (200 mg.) and anhydrous copper sulphate (200 mg.) was heated at 200° for 15 min. The whole was extracted with water and ether, the ethereal solution dried with sodium sulphate and the ether removed. The residue was extracted with boiling alcohol and the solution cooled. The resulting crystals were recrystallized from alcohol and then formed colourless plates, m.p. 263° . This was an ether resulting from loss of 1 mol. of water from 2 mol. of dehydroandrosterone. (Found: C, 81.55; H, 9.7%. $\text{C}_{38}\text{H}_{54}\text{O}_3$ requires C, 81.7; H, 9.75%.) The alcoholic mother-liquors were evaporated to dryness and the residual red gum was distilled at $130^\circ/0.03$ mm. The distillate was twice recrystallized from aqueous methyl alcohol and then formed colourless leaflets, m.p. $87\text{--}88^\circ$, not depressed by the unsaturated ketone isolated from the urine. This sample had $[\alpha]_D^{20} = -31.4^\circ$ ($c = 0.287$ in ethyl alcohol), and the identification was completed by spectroscopic comparison and by the preparation of the

semicarbazone (mixed m.p.). The samples of this ketone from both sources showed very strongly the Rosenheim reaction with trichloroacetic acid, in conformity with the structure assigned to this unsaturated ketone [cf. Schoenheimer & Evans, 1936].

Preparation of 3-androstanone

Dehydroandrosterone semicarbazone (1.1 g.) was heated in a sealed tube at 180° for 18 hr. with sodium ethoxide (1.1 g. of sodium in 15 ml. of alcohol). The product was extracted with ether, dried and distilled. At 120–130°/0.001 mm. an oil distilled which afterwards solidified and was recrystallized from hexane. The crystals (0.6 g.) had an indefinite m.p. (80–100°). This substance was treated with bromine in acetic acid solution; bromine absorption ceased when only 10% of the Br required for the desired carbinol had been added. The whole was then oxidized with chromic acid (0.8 g.) in acetic acid at room temperature (18 hr.), and the product was debrominated by boiling its acetic acid solution with zinc dust. The resulting ketone was purified through its semicarbazone, there being isolated a completely saturated ketone (30 mg.), presumably 3-androstanone (VIII), which crystallized from aqueous methyl alcohol in colourless plates, m.p. 97–98°. (Found: C, 82.7; H, 10.9%. $C_{19}H_{30}O$ requires C, 83.1; H, 11.0%.) The semicarbazone had m.p. 238–240° (from dioxan).

Biological assay

Oestrogenic activity. This was estimated by the procedure of Allen and Doisy. The test animals were ordinary stock mice as supplied by a dealer. The usual precautions were observed to ensure the use only of mice which had been completely ovariectomized, and the animals were shown to give a normal response when injected with oestrone. Sesame oil was used as the medium for the injections and the concentration and spacing of the doses were such that each mouse received 4 doses of 0.1 ml. spread over 30 hr. In order to conserve material only 4 or 5 mice were usually employed for the initial test, but in the case of fractions found to be active the final assay was made with 12 mice. The following results were obtained:

(i) Δ^3 :5-Androstadiene-17-one. The pure unsaturated ketone gave no oestrogenic response when administered to mice in quantities of 1 mg. per mouse.

(ii) Ketone $C_{21}H_{32}O_3$ (?). The analytical sample of the oxime, m.p. 200–202°, was hydrolysed with H_2SO_4 in aqueous alcoholic solution, and the hydrolysate injected into 4 mice so that each mouse received 0.2 mg. No activity was shown. The material obtained from the mother-liquors of the crystallization of this oxime (3 mg.) was found, after hydrolysis, to contain about 50 i.u. of oestrogenic material. This corresponds with 5% of oestrone, a small amount of which might quite conceivably have been carried through into this fraction.

(iii) Semicarbazone of the ketone, $C_{19}H_{28}O_3$, isolated from the "ketonic phenolic" fraction. A specimen of the semicarbazone (6.3 mg.), m.p. 269–270°, which was obtained in the same way as the analytical sample, was hydrolysed and the product injected into 4 mice. It was estimated that this specimen contained about 10–20 i.u. (i.e. the equivalent of 1–2% of oestrone). As the mother liquors of the crystallization of this semicarbazone contained the bulk of the oestrogenic activity of the original urine it is reasonable to assume that the slight activity detected in this less soluble material is due to impurity, and that the pure ketone would be inactive.

(iv) More soluble semicarbazones from the ketonic phenolic fraction. The combined semicarbazones were hydrolysed with H_2SO_4 in aqueous alcohol. The

product gave a strong oestrogenic response and was injected in increasingly high dilutions. It was estimated that this material, from the whole 30 l. of urine, contained about 30,000 I.U., corresponding with 3 mg. of oestrone.

Male hormone activity of $\Delta^{3:5}$ -androstadiene-17-one. The technique used was based on that of Greenwood *et al.* [1935]. In a preliminary test 2 capons received two injections on successive days of a solution of the ketone (0.5 mg.) in sesame oil. The results suggested slight comb-growth activity, and 1 capon was therefore standardized by measuring the comb-growth (length and height) produced by doses of 2 and 3 mg. respectively, of androsterone, and was then given a total amount of 8.5 mg. of $\Delta^{3:5}$ -androstadiene-17-one in two injections on successive days. By the 4th day the increase in size (length and height) of the comb was 11.0 mm. Assuming a linear relationship between the logarithm of the dose and the increase in size of the comb, it was found by interpolation that 1 I.U. (= 100 γ androsterone) was contained in about 350–400 γ of the unsaturated ketone. A high degree of accuracy is of course not claimed, and it is proposed to repeat the assay using artificially prepared material and a larger number of birds.

Test for cortical hormone activity. This was carried out by observing the time of survival of adrenalectomized rats [cf. Schultzer, 1936]. Nine rats of average weight 32 g. were adrenalectomized in one batch and divided into 3 groups (A, B and C) of 3 rats each. Each rat in group A received daily subcutaneous injections of 1 mg. of $\Delta^{3:5}$ -androstadiene-17-one dissolved in 0.1 ml. of sesame oil, the first injection being made on the 2nd day after operation. The rats died after 5.5, 5.75 and 5.9 days, respectively (average 5.7 days). As a control, each of the rats in group B received daily injections of 1 ml. of "Cortin",¹ beginning on the 2nd day after operation. This was continued for 9 days, during which the average weight increased by 10 g., the animals being very active and having excellent appetites. The cortin injections were then discontinued, and each rat then received 1 mg. of $\Delta^{3:5}$ -androstadiene-17-one per day, mixed with the food. Signs of adrenal insufficiency soon appeared, and the rats died 3.5, 5 and 6 days after cessation of the cortin injections (average 4.8 days).

The rats in group C were kept as untreated controls. They died in 5.5, 6.8 and 7.9 days after adrenalectomy (average 6.7 days).

It is clear from these results that the technique was adequate, and that the unsaturated ketone had none of the activity of the cortical hormone, in the doses administered.

SUMMARY

A male patient with a malignant tumour of the adrenal gland excreted an excessive amount of oestrogenic hormone and showed concomitant signs of feminism. An attempt to isolate this hormone from the urine has not been successful, but the results are consistent with the view that the hormone is oestrone. From 30 l. of urine were isolated 0.75 g. of *p*-cresol, and three ketonic compounds which have not hitherto been recognized as urinary constituents. The chief of these three, of which 0.4 g. was isolated, has been shown by analysis and by its artificial preparation by dehydration of dehydroandrosterone to be $\Delta^{3:5}$ -androstadiene-17-one. This ketone had weak comb-growth promoting activity in capons, but had no oestrogenic activity or cortical hormone activity. The other two compounds were isolated only in amounts insufficient for adequate characterization, but analysis of their derivatives suggests that they are represented by the formulae $C_{19}H_{28}O_3$ and $C_{21}H_{32}O_3$. Neither of these had appreciable oestrogenic activity.

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