152. THE ISOLATION OF α-OESTRADIOL AND OESTRONE FROM HORSE TESTES

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ZONDEK [1934] reported the preparation of crude alcoholic extracts of horse testes which possessed a marked oestrogenic potency. Extracts obtained in this way were three hundred times as active oestrogenically as those prepared from a similar weight of mares' ovaries. The high oestrogenic potency of horse testes extracts has been confirmed by later workers. The present investigation was undertaken in order to isolate and identify the oestrogens responsible.

A crude alcoholic extract from 28 kg. of horse testes was generously placed at the disposal of the author by Dr A. S. Parkes. This extract was concentrated, *in vacuo*, the acetone-insoluble material was removed and the acetone-soluble fraction was saponified by refluxing with alcoholic NaOH. The greater part of the oestrogenic activity of the initial concentrate appeared in the alkali-soluble, saponifiable material. That part carried over into the non-saponifiable fraction was recovered by solvent partition. Following the partitioning of the saponifiable fraction between suitable solvents, the concentrate obtained was dissolved in NaOH and the *p*H was adjusted to 9·0. Ether extraction gave a "phenolic" fraction, which on further solvent partition gave 112 mg. of "phenols" possessing practically all the oestrogenic activity of the original "saps". These "phenols" were separated into ketones and non-ketones with Girard's reagent-*T*.

Oestrone was isolated from the ketonic material as the 3:5-dinitrobenzoate (M.P. 188–191° alone or 188–192° when mixed with authentic oestrone 3:5-dinitrobenzoate of M.P. 193–194°). The parent substance (M.P. 238–245°) had the same order of biological activity as authentic oestrone.

α-Oestradiol was isolated from the non-ketones, following digitonin precipitation, as its di-α-naphthoate which melted at 194° alone or when mixed with authentic α-oestradiol di-α-naphthoate (M.P. 194°).

EXPERIMENTAL

All M.P.s are uncorrected. Analyses are by G. Weiler, Oxford.

Bioassays. These were carried out by Dr C. W. Emmens, National Institute for Medical Research, Hampstead, without whose generous co-operation it would have been impossible to have undertaken the investigation. Except for those cases where a group assay is indicated, the dilution method of assay [Beall, 1940] was used. The results were interpreted in terms of oestrone.

Colorimetric assays. When the concentrates had been purified sufficiently their oestrogenic content was estimated by the colorimetric method of Kober [1938]. The colour obtained was read photometrically in an ordinary colorimeter. This was done by placing an Ilford grey screen (0·5 density) against the left-hand

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plunger of the colorimeter and the solution to be read in a micro-cup under the right-hand plunger. An Ilford spectral blue-green filter was placed over the eye-piece and the depth of solution required to give the same intensity of illumination as the standard grey screen was determined [cf. King *et al.* 1937]. Different batches of the β-naphthol reagent gave reproducible results with the same amount of oestrone. A standard curve, using known amounts of oestrone, was accordingly established and the values for the test solutions were read from it.

Preparation of the initial concentrate

The horse testes were minced and extracted in the cold with a relatively large volume of industrial alcohol (10 l./kg.). This extraction was repeated 3 times. The alcohol was decanted off and removed *in vacuo*. The residue obtained was then extracted 4 times with 12 l. portions of boiling acetone. After keeping the acetone solution several days in the ice box the supernatant liquid was decanted and taken to dryness. The residue weighed 387 g.

Concentration of the oestrogenic materials

The acetone-soluble material was saponified by refluxing for 1 hr. with 1 l. of 10% alcoholic NaOH and was separated in the usual way with ether into saponifiable and non-saponifiable fractions. Difficulties due to the formation of emulsions were overcome by the addition of alcohol. The aqueous alkaline solution of "saps" was then acidified and ether-extracted, the ether extract being washed with 2·5% $NaHCO_3$ which removed a large amount of acidic material. In this way 75 g. of "non-saps", 67 g. of "bicarbonate-washed saps" and 108 g. of "acids" were obtained. Bioassays showed that 70% of the total oestrogenic activity of the initial concentrate was in the "saps", the other 30% being divided equally between the "non-saps" and "acids".

The "non-saps" were dissolved in 1 l. of toluene and washed 6 times with 200 ml. portions of *N* NaOH. In each case the aqueous phase, which was partially emulsified, was cleared by centrifuging. The aqueous washings were combined, acidified and ether-extracted, the residue from the ether being added to the "bicarbonate-washed saps". An attempt to wash the toluene phase with water resulted in a very stable emulsion, so it was taken to dryness under reduced pressure, alcohol being added at the final stages to prevent frothing. The residue was taken up in ether, acidified and washed well with water. The ether was taken to dryness, the residue being dissolved in 1 l. of light petroleum and extracted 10 times with 100 ml. portions of 90% MeOH. The alcoholic washings were taken to dryness under reduced pressure and the residue taken up in 1 l. of toluene. The resulting solution was washed 6 times with 100 ml. portions of *N* NaOH. These washings were saturated with CO_2 and ether-extracted. The residue from the ether extract was added to the "bicarbonate-washed saps". In this way all the oestrogenic activity of the "non-saps" was transferred to the "saps".

Attempts to recover the oestrogens in the "acid" fraction were unsuccessful owing to the formation of stable emulsions and this fraction was discarded.

The "saps" were dissolved in 1 l. of light petroleum and extracted 10 times with 100 ml. portions of 90% methyl alcohol. The alcoholic extracts were taken to dryness under reduced pressure and the residue was dissolved in 250 ml. of *N* NaOH. 20 g. of $NaHCO_3$ were added, then *N* HCl was cautiously run in to *p*H 9·0, with vigorous stirring to avoid loss of CO_2. The solution was then extracted 8 times with 250 ml. portions of ether which was taken to dryness to give a "phenolic" fraction. The residue from the light petroleum weighed 33 g.

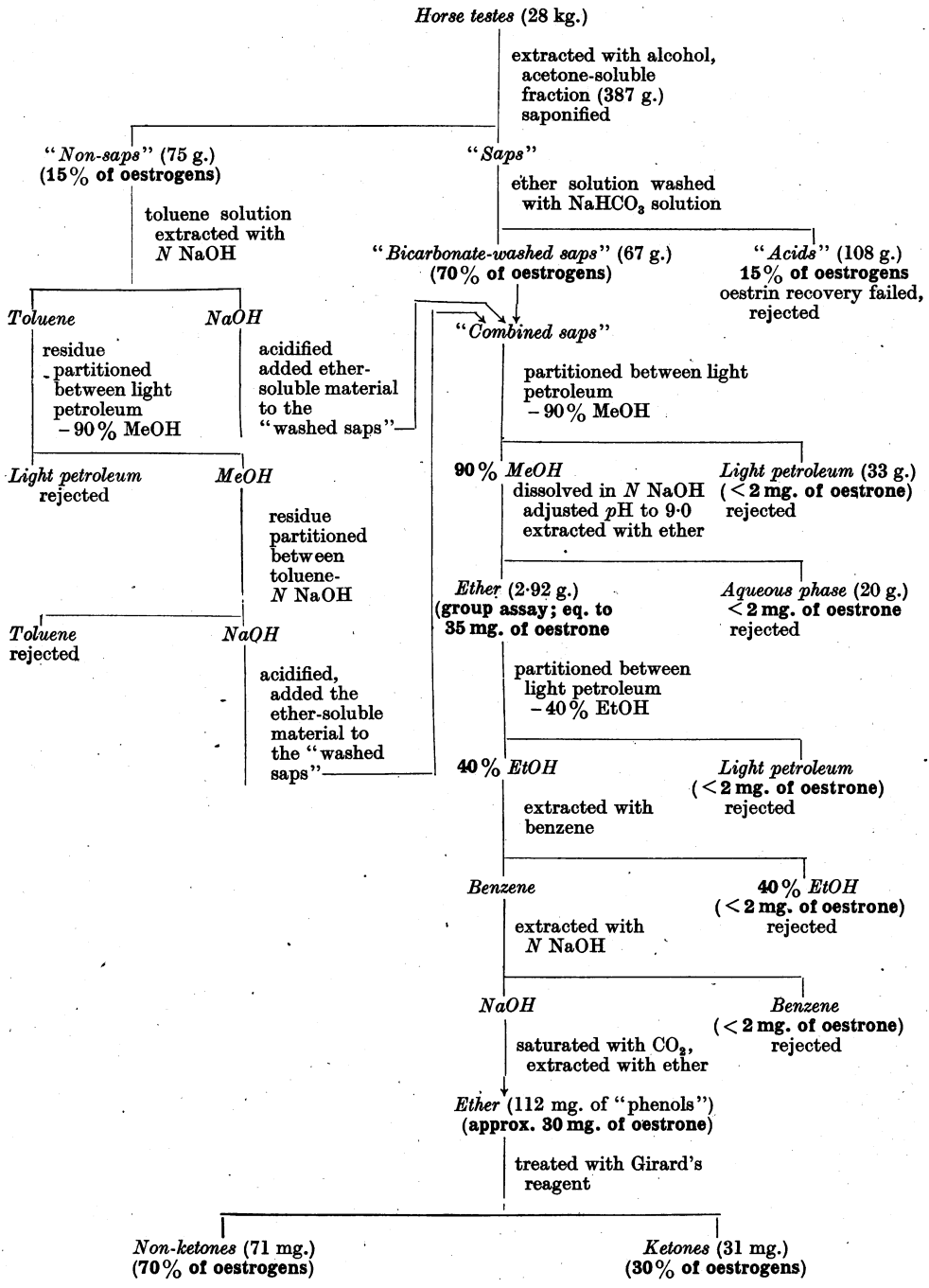

Fig. 1. Flow-sheet of the concentration of the oestrogens.

and contained less than 2 mg. of oestrogen, assayed as oestrone. The ether-soluble material obtained on acidifying the aqueous phase (of *p*H 9·0) weighed 21 g. and contained less than 2 mg. of oestrone. The "phenolic" fraction, which weighed only 2·92 g., had an oestrogenic activity, determined by a group assay, equivalent to 35 mg. of oestrone.

The "phenolic" fraction was dissolved in 100 ml. of light petroleum; 40 ml. of alcohol were added, followed by 60 ml. of water. An opalescent aqueous alcoholic phase separated after shaking. The light petroleum was re-extracted 9 times with 50 ml. portions of 40% alcohol. The combined aqueous alcoholic extracts were then extracted 6 times with 70 ml. portions of benzene which were in turn extracted 6 times with 50 ml. portions of *N* NaOH. The alkaline extracts were saturated with CO_2 till acid to phenolphthalein and ether-extracted. The residue from the ether extract (the "phenols") weighed 112 mg. and contained practically all the oestrogenic activity of the "phenolic" fraction.

The "phenols" were next separated into ketonic and non-ketonic fractions, using Girard's reagent-*T* by the method used in the isolation of oestrone from ox adrenals [Beall, 1940]. Bioassays showed that 70% of the oestrogens were concentrated in the non-ketones and 30% in the ketones. A summary of the partition process is given in Fig. 1.

Isolation of oestrone 3:5-dinitrobenzoate

The ketonic material (31 mg.), which was partially crystalline, was sublimed at 170°/0·05 mm. The sublimate obtained (14 mg.) was crystalline but contained some gum, so it was recrystallized from aqueous alcohol. This gave 3·7 mg. of crystals melting at 238–245°. A bioassay using a group of 10 mice, each receiving 0·06 μg. of these crystals, gave a 10% response. The same response in the control group was elicited by 0·055 μg. of pure oestrone. Colorimetric assay gave results in agreement with those obtained with the same amounts of pure oestrone. The remainder of the crystals was converted into the 3:5-dinitrobenzoate by treatment with 3:5-dinitrobenzoyl chloride in pyridine. The product was crystallized from benzene-alcohol and gave crystals which melted at 188–191° alone or 188–192° when mixed with authentic oestrone 3:5-dinitrobenzoate (M.P. 193–194°). While attempting to sublime the remainder of the crystals *in vacuo* a failure of the pump occurred and the product resinified. Because of this loss of material no analytical figures could be obtained.

Isolation of α-oestradiol di-α-naphthoate

The non-ketones were dissolved in 2 ml. of alcohol and 100 mg. of digitonin in 4 ml. of 50% alcohol were added. The mixture was refluxed for 1 hr. and kept overnight at room temperature. The resulting precipitate was centrifuged, and washed twice by stirring with ether and recentrifuging. The aqueous alcoholic filtrate was diluted with water and ether-extracted, the ether being combined with the ether washings of the digitonin precipitate. The combined ether was washed well with water. Colorimetric assay showed this fraction to contain the equivalent of 3·2 mg. of oestrogens, expressed as oestrone.

The digitonin precipitate was dried, powdered, heated on the water bath for 30 min. with 1·5 ml. of dry pyridine and then poured into 40 ml. of dry ether. The precipitate was filtered off and washed well with ether. It was completely soluble in water, showing that decomposition of the digitonide was complete. The ether was washed with dilute HCl and then water and taken to dryness.

It gave 5 mg. of gum which, assayed colorimetrically, contained the equivalent of 3·7 mg. of oestrone. It was converted into the di-α-naphthoate by the method of MacCorquodale *et al.* [1936], glycine being used to remove the unused acid chloride. The product was taken up in acetone, an equal amount of alcohol was added and the solution was concentrated to 2 ml. A precipitate separated rapidly. After 2 hr. this was collected, redissolved in 2 ml. of acetone and the solvent evaporated under nitrogen, 3 ml. of alcohol being gradually added. The final volume was 2 ml. The resulting crystals weighed 4·5 mg. They softened at 192° and melted at 194° alone or when mixed with authentic α-oestradiol di-α-naphthoate (M.P. 194°). (Found: C, 82·2; H, 6·02%. Oestradiol di-α-naphthoate requires C, 82·7; H, 6·25%.)

Discussion

The "phenols" of the saponifiable material contained the equivalent of 35 mg. of oestrone, estimated by a group assay. Of this 30% (10 mg.) was concentrated in the ketonic fraction and the other 70% (25 mg.) in the non-ketones. By the method of bioassay used α-oestradiol had 4 times the potency of oestrone so that the non-ketones possessed an oestrogenic activity equivalent to 6 mg. of oestradiol. Colorimetric assays of the digitonin precipitate and alcoholic filtrate of this material gave values of 3·7 and 3·2 mg. respectively, a total of 6·9 mg., which is in close agreement with the bioassay results.

That only half the oestrogenic material in the non-ketones was precipitated by digitonin may be due to the fact that α-oestradiol requires a large excess of this reagent for complete precipitation. Unfortunately the alcoholic filtrate from the first precipitation was lost so this point could not be investigated.

On the assumption that the oestrogenic activities of the non-ketonic and ketonic fractions were due to α-oestradiol and oestrone respectively the horse testes examined contained 0·21 mg. of α-oestradiol and 0·36 mg. of oestrone per kg. of wet tissue. This is a much higher concentration than has been reported for other organs. This is illustrated in the data given in the accompanying table.

Organ	α-Oestradiol mg./kg.	Oestrone mg./kg.
Sow ovaries	0·014*	0·010*
Human placentae	0·038†	0·035*
Ox adrenals	—	0·008‡
Horse testes	0·210	0·360

* Westerfeld *et al.* [1938]. † Huffman *et al.* [1940]. ‡ Beall [1940].

The presence of α-oestradiol in horse testes is of interest since it has hitherto been isolated only from tissues and fluids of female origin. Further, this finding prevents the attachment of any idea of sex specificity to the occurrence of this hormone. Speculation as to its path of formation in the male species would be idle with our present limited knowledge of steroid metabolism.

Summary

Using 28 kg. of horse testes α-oestradiol has been isolated (0·21 mg./kg.) as its di-α-naphthoate and oestrone (0·36 mg./kg.) as its 3:5-dinitrobenzoate.

The concentrations of oestrone and of α-oestradiol in horse testes are much higher than the values reported for any other tissue to date.

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