CXXXVII. THE CHEMISTRY OF OESTRIN.

II. METHODS OF PURIFICATION.

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INTRODUCTION.

UNTIL quite recently the lack of an accurate method of assaying oestrus-producing extracts has resulted in most of the work on the preparation and purification of the oestrus-producing hormone being qualitative rather than quantitative.

Workers have been of necessity content with purely empirical methods of preparation and purification, which, although yielding extracts that have been highly potent, have also provided evidence on the chemical nature of the hormone which has undoubtedly been misleading.

Recent publications on methods of assaying the hormone by Coward and Burn [1927], Allan, Dickens, Dodds and Howitt [1928], Kahnt and Doisy [1928] and Marrian and Parkes [1929] have now made quantitative work on the hormone a possibility.

In this communication an attempt has been made to repeat and extend much of the previous work on the stability and purification of oestrin with a view to improving the methods of preparing highly concentrated extracts and throwing more light on the chemical nature of the hormone.

Method of assay.

The method previously described by Marrian and Parkes [1929] has been used throughout this work. Since the publication of this paper numerous tests have been carried out and, provided that the described precautions are observed, the error has been found to be little, if any, more than $\pm 10\%$. No necessity for "priming" the animals as suggested by Kahnt and Doisy [1928] has been found. Provided the animals are injected once a fortnight with not more than 1.5 mouse unit (m.u.) the response is satisfactorily regular.

Standard preparation of oestrin. In a previous communication [Marrian, 1929] the preparation from the urine of pregnancy and initial standardisation of several batches of oestrin were described. These preparations, details of which are shown in Table I, have been used throughout this work.

Table I.

Batch	Type of preparation	Weight of 1 m.u. (mg.)	M.u. per cc. solution
S.B. 1	Aqueous solution	0.0016	37
S.B. 2	Aqueous solution	—	41
E.P.U.	Alcohol-chloroform solution of unsaponifiable matter	0.0183	51
P.U. 4	Unsaponifiable matter	0.00357	—

Samples of the first three batches were used when it was desired only to study the effect of an experiment on the oestrin content of the solution, while batch P.U. 4 was used to investigate the distribution of the total solid matter in relation to the distribution of the hormone in purification processes.

EXPERIMENTAL.

Fractionation with dilute alcohol.

According to several workers, oestrin is soluble to a considerable extent in water-alcohol mixtures [cf. Ralls, Jordan and Doisy, 1926].

If it is assumed that the hormone is a true water-soluble substance, there would seem to be no serious objection to eliminating all the definitely water-insoluble substances by fractionation with alcohol-water mixtures of decreasing strength until only true water-soluble material remains.

The following series of experiments was carried out in order to see how far the dilution of the alcohol could be carried without loss of potency.

(a) 70 % alcohol. Batch P.U. 4, consisting of 1.106 g. of the unsaponifiable ether-soluble fraction from 164 litres of urine, was heated to boiling with 30 cc. of 70 % alcohol for 5 minutes. A considerable amount remained undissolved. The mixture was allowed to stand at 0° for several hours and then filtered. The precipitate on the filter was washed through into the original flask containing the insoluble material with boiling absolute alcohol. After evaporation of the absolute alcoholic solution, the insoluble material was treated twice in the same manner with 15 cc. of 70 % alcohol.

After these three extractions, there finally remained 0.563 g. of insoluble material of which 44 % was cholesterol.

The combined 70 % alcoholic filtrates were evaporated to dryness *in vacuo* and yielded 0.507 g. of a reddish brown semi-solid gum.

Assay. The gum was dissolved in 250 cc. of chloroform. From 1 cc. of this solution the chloroform was removed and the residue made up to 10 cc. with alcohol. After preliminary tests 0.22 cc. of this solution was diluted to 10 cc. with water and injected into a group of mice in four doses of 0.1 cc. each. 58 % of the mice gave a full oestrous response. This corresponds to a dose of 1.07 m.u. per mouse or a total number of 303,900 m.u., the weight of 1 m.u. being 0.00167 mg. Since the original material was assayed to contain 309,500 m.u., it is evident that no detectable loss of activity had occurred, while considerable purification had been effected.

(b) 50 % alcohol. The 70 % alcohol-soluble fraction was treated in an exactly similar manner with 50 % alcohol. In this way 0.335 g. of soluble material and 0.167 g. of insoluble material were obtained. Since only 9 % of the latter consisted of cholesterol, it is probable that the fraction soluble in 50 % alcohol was practically cholesterol-free. This fraction was indistinguishable in appearance from that soluble in 70 % alcohol.

Assay. Working on the assumption that all the activity was in the soluble fraction, the testing was carried out using the same dilutions as in (a). 61 % of a group of mice showed a full oestrous response, corresponding to a total of 315,300 m.u., and a mouse unit weighing 0.00106 mg. Again it was evident that there had been no loss of activity.

(c) 30 % alcohol. By treatment in the same way with 30 % alcohol, 0.133 g. of soluble and 0.206 g. of insoluble material were obtained.

Assay. Preliminary tests showed that the soluble fraction only contained part of the total activity. Both fractions were therefore made up to 250 cc. in alcohol. 1 cc. of the soluble fraction was diluted to 10 cc. with water and injected in four doses of 0.1 cc. each. 20 % of a group of mice gave an oestrous response, corresponding to 0.83 m.u. per mouse, or a total of 86,450 m.u. The weight of one mouse unit of this fraction was therefore 0.00154 mg.

0.3 cc. of the solution of the insoluble fraction was diluted to 10 cc. with water and injected in four doses of 0.1 cc. each. Oestrus was shown by 60 % of the group of mice. This corresponds to 1.09 m.u. per mouse and a total of 227,000 m.u. The weight of one mouse unit of this fraction was 0.000865 mg.

The total mouse units in each fraction add to 313,450 which is in satisfactory agreement with the original amount of 315,300 m.u. This result could be explained in one of two ways, either the hormone has only a low solubility in 30 % alcohol or it has a high solubility but is readily adsorbed by filter-paper from 30 % alcohol. In the latter case, which seems possible in view of the statement of Laqueur, Hart and de Jongh [1927] that oestrin is readily adsorbed by filter-paper from aqueous solutions, the adsorbed hormone would be washed back with the insoluble fraction on washing the filter with boiling alcohol. In either case, it is evident that fractionation with 30 % alcohol does not provide an efficient means of purification.

Resistance to alkali treatment and extraction from aqueous media by ether.

That oestrin may resist boiling for long periods with strong aqueous alkalis or sodium ethoxide solutions seems to be clear from the work of Ralls; Jordan and Doisy [1926] and Laqueur, Hart and de Jongh [1926], although the former experienced losses which they consider may possibly have been due to causes other than hydrolysis. The following experiments entirely confirm these findings.

(a) *Aqueous potassium hydroxide.* 1 cc. of batch S.B. 1 was placed in each of two 10 cc. volumetric flasks. 5 cc. of 4.8 % aqueous KOH were added, and the flasks were heated for different periods in a boiling water-bath. To avoid

possible losses by oxidation, a stream of nitrogen was blown over the surface of the liquid. At the end of the experiment, after cooling and exactly neutralising with the predetermined quantity of acetic acid, the contents were made up to 10 cc. These solutions were then injected into groups of mice in four doses of 0.1 cc. each. The results are shown in Table II.

Table II. *Resistance of oestrin to hot aqueous KOH.*

M.u. in each flask before experiment	Time heated (mins.)	Mice on oestrus (%)	M.u. in each flask after experiment
37	40	75	35
37	180	82	39

(b) *Sodium ethoxide.* In a second series of experiments, 4.5 cc. of batch E.P.U. were placed in each of two flasks. The solvent was removed by evacuation of the flask at 100° and 5 cc. of 0.2 *N* sodium ethoxide solution were added to each flask. The flasks were then heated on a hot plate to a temperature of 100° under reflux condensers for different times, a stream of nitrogen being played on the surface of the liquid. After neutralisation with the exact amount of acetic acid, the volumes were made up to 60 cc. with water and four doses of 0.1 cc. injected into two groups of mice. The results are shown in Table III.

Table III. *Resistance of oestrin to hot Na ethoxide.*

M.u. in each flask before experiment	Time heated (mins.)	Mice on oestrus (%)	M.u. in each flask after experiment
216	40	85	246
216	120	75	204

These results show quite definitely that oestrin is quantitatively stable to treatment with aqueous alkalis and sodium ethoxide at 100° for at least 2 hours. This does not necessarily mean, however, that crude preparations of the hormone can be satisfactorily purified by ether extraction of the saponified material. On the contrary a considerable amount of evidence is accumulating to show that such a procedure may result in the loss of a considerable fraction of the total activity.

Thus Allan, Dickens, Dodds and Howitt [1928] show that the washing of an ethereal solution of the hormone by aqueous alkali results in a partition of the activity between the two layers. They state that this is due to adsorption by alkali soaps in the aqueous layer. Glimm and Wadehn [1929] claim that boiling with alkali converts the hormone into a form insoluble in ether. Funk [1929] states that oestrin is alcoholic or phenolic in nature and on treatment with alkalis forms salts. He explains the appearance of the hormone in the unsaponifiable matter on the grounds that the alkali salts are soluble to some extent in ether.

Accordingly it was decided to study quantitatively the extraction of oestrin from aqueous media at neutral, acid and alkaline reactions. It is extremely

important to use only freshly redistilled ether of the highest degree of purity for such extractions. Numerous results have been frequently obtained confirming the statements of Thayer, Jordan and Doisy [1928] that the hormone is rapidly destroyed by ether peroxides.

(c) *Extraction of neutral aqueous solution by ether.* 50 cc. of batch S.B. 2 containing 2050 m.u. were shaken in a funnel with about 80 cc. of ether in four successive quantities of 20 cc. The combined ethereal extracts were washed once with about 20 cc. of water and evaporated to dryness. The residue was dissolved in about 3 cc. of alcohol and then made up to 50 cc. with water. 0.66 cc. of this solution was diluted to 10 cc. and after injection into a group of mice in four doses of 0.1 cc., produced a 60 % oestrous response. This corresponds to a total of 2065 m.u. Thus the extraction of the hormone from neutral solution by ether was complete.

(d) *Extraction of acid aqueous solution by ether.* 5 cc. of E.P.U. solution containing 255 m.u. were evaporated to dryness and then dissolved in about 1 cc. of alcohol. 80 cc. of 0.5 N HCl were added and the mixture was shaken out four times with 30 cc. of ether. The combined ethereal extracts, after washing once with 20 cc. of water, were evaporated to dryness, dissolved in 5 cc. of alcohol and made up to 100 cc. This solution was injected into a group of mice in four doses of 0.1 cc. and 35 % of the group showed an oestrous response. This corresponds to a total of 230 m.u., and, within the limits of error of testing, may be considered to represent quantitative recovery of the hormone.

(e) *Extraction of alkaline aqueous solution by ether.* 5 cc. of E.P.U. were evaporated to dryness and dissolved in a small volume of alcohol. 80 cc. of 4.8 % aqueous KOH were added and the mixture was then extracted four times with 30 cc. of ether. The combined ethereal extracts were washed once with 20 cc. of water and evaporated to dryness. The residue was dissolved in 3 cc. of alcohol and made up to 100 cc. with water. This was injected in four doses of 0.2 cc. each. The group of mice showed a 20 % positive response, corresponding to a total of 103 m.u.

Since it was expected that ether extraction from alkaline solution might prove to be incomplete, the alkaline solution, together with the washing of the ether extract, was acidified with 6 cc. of 12 N HCl and re-extracted with ether as before. The product was made up to 100 cc. as in the previous experiments and injected in four doses of 0.15 cc. each. This resulted in a 30 % oestrous response or a recovery of a further 148 m.u. The total recovery of 251 m.u. is quantitative.

The failure of oestrin to be extracted with ease from aqueous potassium hydroxide solutions by ether would seem to be due to either (a) the formation of a potassium salt which is slightly soluble in ether as suggested by Funk [1929], (b) the formation of a potassium salt which dissociates to some extent, thus allowing the free dissociated oestrin to dissolve in the ether layer, or (c) adsorption by soaps in the aqueous layer, as suggested by Allan, Dickens,

Dodds and Howitt [1928]. The last possibility seems somewhat improbable as the batch E.P.U. was prepared by a saponification process [Marrian, 1929] and should be reasonably free from soaps or easily saponifiable substances. It will be shown in a later experiment that this loss is almost certainly not due to adsorption by soaps.

Since the evidence indicated that oestrin might be acidic in nature as Funk suggests, it was thought desirable to ascertain what effect in the extraction by ether a preliminary saturation of the alkaline solution by carbon dioxide would have. 5 cc. of E.P.U. were treated in a manner identical to that in the last experiment with the exception that carbon dioxide was bubbled through the alkaline solution for 1.25 hours before ether extraction. The residue from the ether extraction was made up to 100 cc. as before and injected in four doses of 0.2 cc. each. An oestrous response of 89 % corresponding to a recovery of 229 m.u. resulted.

This result suggests that oestrin is a weakly acidic substance which can be displaced from combination with alkalis by carbon dioxide, and that the loss on purification by a saponification process might be avoided by such treatment.

Extraction of oestrin from ethereal solution by aqueous alkali.

The low solubility in ether of what may provisionally be called the potassium salt of oestrin and its higher solubility in aqueous alkalis suggested the possibility of completely removing the active principle from an ethereal solution by repeated extraction with aqueous KOH. Since all the preparations used had been prepared by the converse process, *i.e.* extraction from aqueous alkaline solution by ether [Marrian, 1929], it was hoped that considerable purification might be effected in this manner.

In a preliminary experiment, 5 cc. of E.P.U. solution were evaporated to dryness and dissolved in 80 cc. of ether. This solution was shaken four times in succession with 30 cc. of 4.8 % KOH. The combined potash extracts were approximately neutralised with HCl and the ether was removed by evaporation under reduced pressure. The solution, after exact neutralisation to litmus with acetic acid, was made up to 200 cc. and injected in four doses of 0.25 cc. 63 % of the mice gave an oestrous response which corresponds to a recovery of 226 m.u. This experiment clearly shows that from such an ethereal solution the hormone can be completely extracted by aqueous potassium hydroxide.

It was still possible, however, that this might be due to adsorption of the active principle by traces of potassium soaps; accordingly it was decided to repeat the experiment on batch P.U. 4 which was certainly free from soaps or readily saponifiable substances, since during its preparation it had been saponified twice and the soaps had been removed by frequent washing with water [Marrian, 1929]. This experiment would also serve to study the distribution of total solids between the two phases, so that the efficiency of the process as a method of purification could be investigated.

The two fractions of P.U. 4 (soluble and insoluble in 30 % alcohol) were

recombined and made up to a known volume. An aliquot portion was removed for physiological tests, leaving on evaporation 290,000 m.u. and 0.308 g. of solid material. This was dissolved in 500 cc. of ether and extracted in a funnel with seven successive lots of 100 cc. of 4.8 % KOH. The combined alkali extracts, after acidification with 80 cc. of 12N HCl, were extracted twelve successive times with 100 cc. of ether. The combined ethereal extracts, after washing twice with water, were evaporated to dryness and yielded 0.0265 g. of a dark reddish semi-solid that appeared to contain a certain amount of crystalline material.

This was made up to 250 cc. with chloroform. 0.15 cc. of this solution was evaporated to dryness and, after dissolving the residue in 5 cc. of alcohol, this solution was made up to 50 cc. with water. Two groups of mice were injected with the solution in four doses of 0.1 cc. and four doses of 0.15 cc. These two groups showed oestrous responses of 30 % and 80 % respectively, corresponding to a total of 205,100 and 187,500 m.u. From the average of these figures the weight of one mouse unit is 0.000135 mg.

The stages in the purification of batch P.U. 4 are presented in Table IV.

Table IV. *Purification of oestrin: batch P.U. 4.*

1.106 g. unsaponifiable matter from 164 litres of urine: 309,500 m.u. 1 m.u. = 0.00357 mg.	
70 % C ₂ H ₅ OH	
Soluble 0.507 g. 303,900 m.u. 1 m.u. = 0.00167 mg.	Insoluble 0.563 g. 44 % cholesterol: not tested
50 % C ₂ H ₅ OH	
Soluble 0.335 g. 315,300 m.u. 1 m.u. = 0.00106 mg.	Insoluble 0.167 g. 9 % cholesterol: not tested
30 % C ₂ H ₅ OH	
Soluble 0.133 g. 86,450 m.u. 1 m.u. = 0.00154 mg.	Insoluble 0.206 g. 227,000 m.u. 1 m.u. = 0.00086 mg.
Incomplete separation; recombined	
Sample of 0.0267 g. removed 0.308 g. 290,000 m.u. 1 m.u. = 0.00106 mg.	
Dissolved in ether, extracted with alkali. Ether-soluble material extracted from alkali after acidification	
Alkali-soluble 0.0265 g. 196,300 m.u. 1 m.u. = 0.000135 mg.	Alkali-insoluble 0.283 g. 27,080 m.u. 1 m.u. = 0.0105 mg.

The original ether solution, after extraction by the alkali, was washed once with water and evaporated to dryness. 0.2833 g. of a dark red-brown gum was obtained, differing from the other fraction in not appearing to contain any crystalline material. This gum was dissolved in chloroform and made up to a volume of 250 cc. 1.2 cc. of this solution were evaporated to dryness and, after dissolving the residue in 5 cc. of alcohol, this was diluted to 50 cc. with water. A group of mice injected with four doses of 0.1 cc. gave a 55 % positive response, corresponding to a total of 27,080 m.u. in this fraction.

The total recovery of 223,380 m.u. represents a loss of just over 20 %. This is probably well outside the maximum error in the method of assay and must be considered to be experimental loss. Although the separation was not quite so complete as was at first hoped for, the method clearly provides a most efficient means of removal of inactive material from the extracts.

Solubilities of main oestrin fraction.

Attempts were made to obtain material which was definitely crystalline from the oestrin concentrate of 0.0265 g. by recrystallisation. So far, however, no suitable solvent has been found.

The material was extremely soluble in methyl, ethyl and propyl alcohols, chloroform, acetone and ethyl acetate. In ether, benzene, hexane, 30 % alcohol and water the solubility appeared to be extremely small. Laqueur, Hart and de Jongh [1927], working with concentrates obtained from placenta, state that the purest preparations of the hormone (0.0008 mg./m.u.) which they were able to obtain were no longer soluble in the common volatile fat solvents but only in water. In view of these somewhat conflicting observations it must be concluded that present data on the solubilities of the concentrates give little reliable evidence on the actual solubilities of the hormone itself. The observed solubilities must be considered as properties merely of the substances with which the hormone is associated, the nature of which must vary with the method of preparation and the source.

Doisy, Veler and Thayer [1929] found that their method of preparing aqueous solutions of the hormone from extracts of liquor folliculi resulted in failure with urine concentrates. This method, consisting of solution of the concentrate in alcohol, dilution with water and subsequent removal of the alcohol by evaporation under reduced pressure, is essentially the same as that adopted by Allan, Dickens, Dodds and Howitt [1928].

An attempt to prepare an aqueous solution from the main oestrin fraction of 0.0265 g. by this method was only partially successful. After boiling off the alcohol, a large amount of solid material separated. On assaying the oestrin content of the clear supernatant liquid, it was found that about two-thirds of the total activity must have been present in the separated solid. Unfortunately Doisy, Veler and Thayer do not state the exact nature of the

difficulties encountered, but since their product was approximately of the same potency¹ as the one here described, it may be assumed that they were similar.

At present no satisfactory quantitative method of obtaining the activity from urine extracts in a "water-soluble" form has been devised.

SUMMARY.

1. Using the method of assay of Marrian and Parkes [1929] a study of the efficiency of different methods of purifying oestrus-producing extracts from urine has been made on a quantitative basis.

2. By fractionation with 70 % and 50 % alcohol, the activity may be obtained quantitatively in the soluble fraction in each case, with considerable loss of the total solid matter.

3. The high degree of stability of the hormone to boiling aqueous alkalis and sodium ethoxide, found by numerous other workers, has been confirmed.

4. Oestrin can be extracted quantitatively from neutral or acid aqueous solutions by ether with ease, but it is much less readily extracted from alkaline aqueous solution.

5. After passing carbon dioxide into an aqueous alkaline solution of oestrin, the hormone may be quantitatively extracted by ether with ease. This suggests that oestrin is a weakly acidic substance.

6. Oestrin may be extracted from an ethereal solution by aqueous potassium hydroxide. This process removes almost all the activity from the ethereal solution and only a small proportion of the solid matter.

7. By these means a concentrate with an activity of 1 m.u. = 0.000135 mg. has been prepared. The solubilities of this preparation are described.

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REFERENCES.

- Allan, Dickens, Dodds and Howitt (1928). *Biochem. J.* **22**, 1526.
Coward and Burn (1927). *J. Physiol.* **63**, 270.
Doisy, Veler and Thayer (1929). Abstracts Comm. XIIth Internat. Physiol. Congr.
Funk (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 568.
Glimm and Wadehn (1929). *Biochem. Z.* **207**, 361.
Kahnt and Doisy (1928). *Endocrin.* **12**, 760.
Laqueur, Hart and de Jongh (1926). *Deutsch. med. Woch.* **52**, 1331.
—— (1927). *Lancet*, i, 1126.
Marrian (1929). *Biochem. J.* **23**, 1090.
—— and Parkes (1929). *J. Physiol.* **67**, 389.
Ralls, Jordan and Doisy (1926). *J. Biol. Chem.* **69**, 357.
Thayer, Jordan and Doisy (1928). *J. Biol. Chem.* **79**, 53.

¹ They report the preparation from urine of an extract, the rat unit of which is less than 0.001 mg. Since the rat unit is probably from seven to ten times greater than the mouse unit, this preparation must be of about the same degree of potency as the one described in this paper.