

CXVII. THE CHEMISTRY OF OESTRIN.

I. PREPARATION FROM URINE AND SEPARATION FROM AN UNIDENTIFIED SOLID ALCOHOL.

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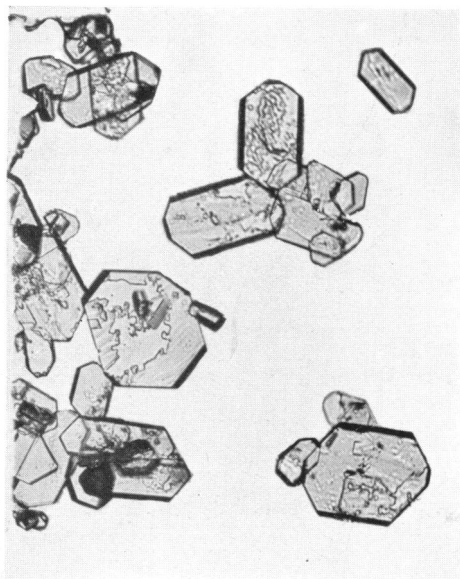
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INTRODUCTION.

ASCHEIM and ZONDEK [1927] showed that the urine of pregnant women contained large amounts of oestrin which could readily be extracted by ether or chloroform. The yields obtained were about 1000 mouse units per litre, which weight for weight compares very favourably with yields from ovaries or placentae. In a later paper [1928] they showed that yields could be obtained as high as 10,000 mouse units per litre. Since urine usually contains only a relatively small amount of ether-soluble material, the crude product so obtained is considerably more potent than the crude ether-soluble material from other sources. In addition the ready accessibility of the raw material and the economy in labour and solvents make this the most convenient starting material for a chemical attack on oestrin. The urine of pregnancy has since been used as a source of oestrin by several other workers [Slotta, 1927; Veler and Doisy, 1928; Glimm and Wadehn, 1929; Marrian and Parkes, 1929].

As a preliminary to such a chemical attack, several preparations have been made, by purely empirical methods that are known to yield active products, and accurately standardised, so that previous work on the stability and purification of the hormone could be repeated and extended in a strictly quantitative manner.

Essentially, the process in most cases consisted of a brief aqueous saponification of the ether-soluble material from the urine and subsequent extraction of the unsaponifiable matter with ether. This unsaponifiable matter contained varying quantities of a white crystalline solid, superficially very similar in appearance to cholesterol but, unlike cholesterol, only very slightly soluble in ether. This property provided an easy means of removing the more soluble pigmented fraction containing the oestrin. By recrystallisation of the solid from boiling acetone, perfectly white, crystalline plates were obtained which melted at 233–235°. On microscopic examination this appeared to be a single substance with a characteristic crystalline form (Plate VI).



The alcohol isolated from urine of pregnancy

The substance could not be identified in the unsaponifiable matter from batch S.B. 2, but in this case chloroform was used throughout the process instead of ether, as a result of which large amounts of caffeine appeared in the final product. Since the melting-points and solubilities of this substance and caffeine are very similar, separation was impracticable, while identification by microscopic examination failed owing to the large excess of the latter substance.

However, the substance was detected with unfailing regularity in all the numerous sub-batches of the subsequent large batches and there can be no doubt that it is a usual constituent of the unsaponifiable matter of such urines. The question of its specificity to pregnancy will be discussed in a later section.

The present communication describes in detail the preparation and standardisation of these extracts, and includes the results of an investigation of the chemical nature of this unknown substance.

The method of assay described in a previous paper [Marrian and Parkes, 1929] has been used throughout this work.

The urine was collected daily from patients one or two weeks before parturition.

PREPARATION AND STANDARDISATION OF EXTRACTS.

Batch S.B. 1. This was a water-soluble extract prepared from 19 litres of urine by a method based on that of Zondek and Brahn [1925]. The details have been described previously [Marrian and Parkes, 1929].

Batch S.B. 2. 155 litres of urine were collected in vessels containing a small quantity of chloroform and sulphuric acid. The urine was evaporated in open basins to about a quarter of the original volume and extracted 6–8 times with chloroform. This extract was evaporated to dryness and then heated for 30 minutes with 800 cc. of 5 % alcoholic KOH. Most of the alcohol was then removed by evaporation under reduced pressure and, after dilution with water, the mixture was extracted eight times with chloroform. The chloroform extract, after washing six times with water, was evaporated to dryness *in vacuo*. The product was a reddish-brown gum and weighed 5.01 g. By extraction of this with 25 cc. of ice-cold ether, 0.53 g. of insoluble crystalline material remained behind. This subsequently proved to be impure caffeine.

The ether-soluble material was then resaponified with 50 cc. of 5 % alcoholic KOH, diluted with water and re-extracted eight times with chloroform. The residue from this extract after thorough washing with water was extracted ten times with 50 cc. 0.1 *N* acetic acid in a boiling water-bath for 10 minutes. Each acid extract was filtered through a pad of glass wool, the residual unsaponifiable matter being redissolved in chloroform and evaporated to dryness each time before the next addition of acid in order to expose a fresh surface for extraction.

The combined extracts were neutralised to p_H 6.8 with NaOH, made up to 1 litre with water, and boiled, 0.1 % “tri-cresol” being added before boiling as a preservative.

The product was a slightly yellow opalescent solution.

Assay of S.B. 2. 0.5 cc. was diluted to 10 cc. with water. 0.4 cc. of this was injected into each of 20 mice in four doses of 0.1 cc. spread over 36 hours. Four mice, or 20 %, gave a positive vaginal reaction as previously defined [Marrian and Parkes, 1929], corresponding to a dose of 0.82 mouse unit per mouse. The solution therefore contained 41 mouse units per 1 cc.

Batch E.P.U. 21 litres of urine were collected without any preservative and extracted six times with ether. The extract was washed six times with water, and evaporated to dryness. This residue was saponified by heating on a boiling water-bath for 10 minutes with 200 cc. 4 % KOH, cooled, diluted with water and extracted with ether eight times. The extract after washing eight times with water was evaporated to dryness and yielded 0.257 g. of a yellow gummy material mixed with a white crystalline solid. On treating the product with 5 cc. of ice-cold ether, the white solid was left undissolved and was filtered off. This was washed with a further 5 cc. of ether and the combined ethereal solutions were evaporated to dryness. The gummy residue, weighing 0.233 g., was dissolved in a mixture of alcohol and chloroform and made up to 250 cc. The addition of chloroform was necessary as the material seemed to be partly insoluble in alcohol alone.

Assay of E.P.U. 0.64 cc. alcohol-chloroform solution was diluted to 10 cc. with water after boiling off the chloroform. The resulting suspension was stable and quite suitable for injection. Four doses of 0.1 cc. produced a 75 % and 70 % vaginal response in two groups of 20 mice. These correspond to doses of 1.34 and 1.25 mouse units per mouse, respectively, *i.e.* 52.3 and 48.8 mouse units per 1 cc. of original alcohol-chloroform solution: mean, 51 mouse units per 1 cc.

Batch P.U. 4. This batch of 164 litres was worked up in a number of small sub-batches of about 20 litres each, so that the effect of variations in the method of extraction on the yield of unsaponifiable matter and of the unknown substance could be studied. As a result of this the following method was adopted and was used for the greater part of the batch.

The urine was collected daily in vessels containing a small amount of toluene as a preservative. It was then extracted in large funnels, once with toluene and three times with ether. The toluene and ether extracts were evaporated to a small bulk, combined, and stored in alcohol until it was convenient to proceed with the saponification.

When about 20 litres of urine had been so treated, the alcohol was evaporated from the combined extracts and the residue heated for 10 minutes with 200 cc. aqueous 4 % KOH on a boiling water-bath. The mixture was then cooled, diluted, and extracted with ether eight times. The addition of a small quantity of toluene to the ether used in these extractions greatly facilitated the breaking of otherwise very troublesome emulsions. The total extract was washed eight times with water to remove soaps and traces of alkali, and evaporated to dryness. The last traces of water were removed by evacuation

of the flask for half an hour at 100°. The unsaponifiable matter obtained in this way varied much in amount and appearance, even with the same method of preparation. In some cases the residue consisted almost entirely of a white crystalline solid, in others of a small amount of light yellow gummy matter together with a little crystalline solid. Often the product was highly coloured with red pigments. Invariably, however, these products contained a small amount of solid, relatively insoluble in ether, which had the same characteristic crystalline form as the first preparation. The total amount of unsaponifiable matter obtained in this way was 2.069 g. This was extracted with 50 cc. of ice-cold ether, filtered and the insoluble material washed with a further 5 cc. of ether.

The ether-soluble material, weighing 1.611 g., was resaponified by heating to 100° for 0.5 hour with 0.5 g. sodium in 30 cc. alcohol. After dilution with water, the mixture was extracted with ether six times. The ether extract after being washed six times with water was evaporated to dryness and yielded 1.106 g. of a clear reddish-brown gum. This material was stored in chloroform.

Assay of P.U. 4. The total unsaponifiable matter was made up to 250 cc. in chloroform. 1 cc. of this was diluted to 10 cc. with alcohol and then 0.22 cc. of this alcoholic solution to 10 cc. with water. This injected in four doses of 0.1 cc. produced a positive vaginal reaction in 60 % of a group of 20 mice, corresponding to a dose of 1.09 mouse units per mouse. Thus the total number of mouse units obtained was 309,500, the weight of 1 mouse unit being 0.00357 mg.

Batch P.U. 5. 164 litres of urine were extracted by the method used in the case of P.U. 4 in order to obtain a larger quantity of the unknown substance for chemical examination. The oestrin fraction has not yet been standardised. Before separation of the unknown substance, the crude unsaponifiable matter was resaponified with sodium ethoxide. The total unsaponifiable matter weighed 4.400 g. On account of persistent clogging of the filter it was not practicable to effect a separation by means of ether as in the previous batches. Two extractions with ice-cold acetone, however, removed the more soluble oestrin fraction, leaving an insoluble residue from which the substance was subsequently separated.

A summary of the yields and weights of mouse units from the different batches is shown in Table I.

Table I.

Batch No.	Volume of urine litres	Mouse units per 1 litre urine	Weight of 1 mouse unit mg.	No. of mouse units per 1 cc. final solution
S.B. 1	19	974	0.0016	37
S.B. 2	155	265	—	41
E.P.U.	21	607	0.0183	51
P.U. 4	164	1887	0.00357	—

THE UNIDENTIFIED COMPOUND.

Purification. The ether-insoluble material was extracted in each case with several successive amounts of boiling acetone and the solution filtered hot. A small amount of white amorphous material, the nature of which has not been investigated, remained undissolved. In the case of batch P.U. 5, where the separation was effected in the first place with cold acetone, a bulky red amorphous substance remained undissolved in the boiling acetone. On cooling the acetone solution perfectly white crystalline plates were deposited. These were filtered and again crystallised from boiling acetone. The melting-points of the finally purified products from batches P.U. 4 and P.U. 5 were 232–233° and 233–234.5° (uncorrected). In both cases the substance melted sharply and no sign of decomposition was observed. The presence of the hot acetone-insoluble impurity could be detected in impure specimens by a darkening of the substance a few degrees below its melting-point.

The yields of unsaponifiable matter and of the compound (X) are shown in Table II.

Table II.

Batch No.	Volume of urine litres	Weight of unsap. matter g.	Weight of	Weight of X g.	Weight of X per 100 litres g.
			unsap. matter per 100 litres g.		
S.B. 1	19	0.275	1.447	0.0415	0.218
E.P.U.	21	0.257	1.224	0.0041*	0.019
P.U. 4	164	2.069	1.261	0.2133*	0.130
P.U. 5	164	4.400	2.683	0.1420	0.087

* The impurity insoluble in hot acetone was not removed before weighing.

Analysis and molecular weight. Nitrogen, sulphur and halogens were absent. Micro-combustions on samples from batches P.U. 4 and P.U. 5 gave the following results:

X (batch P.U. 4) 4.480 mg. gave 12.810 mg. CO₂ and 4.480 mg. H₂O.

C = 77.97 %, H = 11.11 %.

X (batch P.U. 5) 4.941 mg. gave 14.170 mg. CO₂ and 5.000 mg. H₂O.

C = 78.20 %, H = 11.24 %.

Molecular weight determinations were carried out by Rast's camphor method. The results are summarised in Table III.

Table III.

	X (average figures)	Calculated for		
		C ₁₈ H ₂₂ O ₂	C ₁₉ H ₂₂ O ₂	C ₂₀ H ₂₄ O ₂
C	78.08 %	77.14 %	78.07 %	78.43 %
H	11.18 %	11.43 %	10.95 %	11.11 %
O	10.74 %	11.43 %	10.95 %	10.45 %
M.W.	284	280	292	306
(av. of 4 results)				

The combustion results indicate that the compound has a C₁₉ or C₂₀ formula, although the molecular weight determinations appear to be more in agreement

with $C_{18}H_{32}O_2$. However, the error of micro-molecular weight determination by this method on a compound of such a high molecular weight may be considerable, and thus one or other of the two higher formulae is considered to be more probable.

Solubility and general properties. One of the most striking characteristics of the substance is its low solubility in all the common organic solvents. Its solubility in ether although extremely low is nevertheless sufficient to make it possible for it to be extracted completely from the saponified mixture if fairly large volumes are used. Its solubility in light petroleum and in cold acetone is also very slight. In cold benzene, toluene, ethyl alcohol, methyl alcohol, chloroform, the solubility is very much greater, but rough quantitative determination showed it to be less than 1 % in each case. On heating, the solubility in these solvents is increased to some extent. In boiling acetone the solubility is fairly high and this solvent is perhaps the most convenient to employ for recrystallisation. The substance is completely insoluble in hot or cold water, or aqueous alkalis.

The iodine value of the substance was determined on 8 mg. by a micro-modification of Dam's [1924] method. No absorption of bromide occurred; the substance is therefore saturated.

A few of the colour reactions characteristic of the sterols were tried. Salkowski's was completely negative. Lipschutz's so-called "oxycholesterol" reaction with benzoyl peroxide, acetic and sulphuric acids was also negative. The Liebermann-Burchardt reaction with acetic anhydride and H_2SO_4 in chloroform solution gave a deep red-brown colour. This reaction which was positive for every batch is very sensitive and is given strongly by a trace of the compound. The substance was not precipitated from hot alcoholic solution by an alcoholic solution of digitonin.

Attempts to determine the specific rotation have so far been unsuccessful owing to the difficulty in obtaining anything other than a very dilute solution in any of the usual solvents.

The pure substance had no oestrus-producing activity as shown by the following experiment. A weighed quantity was dissolved in a minimal quantity of boiling alcohol. A small quantity of water was then added and the alcohol boiled off. On shaking, an emulsion suitable for injection resulted. This was injected into three ovariectomised mice in four doses spread over 36 hours, so that two received a total amount of 0.13 mg. and one 0.26 mg. The vaginal smears were completely negative.

Alcoholic nature: preparation of the acetate.

The presence of two oxygen atoms in the molecule suggested that the substance might be an alcohol. Its low solubility in ether and its relatively greater solubility in methyl or ethyl alcohol would be in keeping with the presence of two hydroxyl groups. Accordingly an attempt was made to prepare an acetate. 0.0585 g. of the substance (batch P.U. 4) was heated to

100° for 2 hours with 5 cc. of acetic anhydride, in which it readily dissolved. Excess of water was then added and after scratching for some time with a glass rod a perfectly white solid separated out. This was filtered off and washed thoroughly with water to remove excess of acetic anhydride and yielded 0.0684 g. of the crude acetate.

In a second experiment 0.0804 g. of the substance from batch P.U. 5 yielded 0.1037 g. of the crude acetate. If the substance is a dihydroxy-alcohol of the formula $C_{19}H_{30}(OH)_2$ then 292 g. (1 mol) should yield 376 g. of the diacetate, $C_{19}H_{30}(OCOCH_3)_2$. If only one oxygen atom is present as hydroxyl the corresponding yield should be 334 g. Actually the yields of the two experiments described above work out respectively at 343 g. and 378 g. per 1 mol. These figures suggest that both oxygen atoms are present as hydroxyl groups.

Attempts to estimate the number of hydroxyl groups in the alcohol by a micro-modification of the method of Hibbert and Sudborough [1904] failed owing to the insolubility of the alcohol in the amyl ether used. All efforts to overcome this difficulty by the use of other suitable solvents with a low vapour pressure have failed for the same reason.

The acetate was found to have a much greater solubility in the common organic solvents than had the parent alcohol. It was readily soluble in the cold in ethyl alcohol, chloroform and acetone. It was found, however, to be only very slightly soluble in cold methyl alcohol, but readily soluble on heating. The acetate by this means could be easily recrystallised. After two such recrystallisations the preparations were considered to be fairly pure.

The preparation of acetate from batch P.U. 4 before the first crystallisation melted to an opaque liquid at 158–162°. After one recrystallisation a partial melting was observed at 160–163°. On raising the temperature no change was observed up to 177° when it melted completely. After the second recrystallisation the substance melted sharply and completely at 177.5–178.5°.

The preparation from batch P.U. 5 still showed this double melting-point even after two recrystallisations.

Analyses and molecular weight.

Batch P.U. 4. 2.835 mg. gave 7.690 mg. CO_2 and 2.51 mg. H_2O .

C = 73.96 %, H = 9.84 %.

Batch P.U. 5. 5.100 mg. gave 13.875 mg. CO_2 and 4.62 mg. H_2O .

C = 74.20 %, H = 10.06 %.

m.w. (Rast): 358, 327. Mean: 343.

Calculated for $C_{18}H_{30}(OCOCH_3)_2$:	C = 72.52 %	H = 9.89 %	m.w. = 364.
„ „ $C_{18}H_{31}O.OCOCH_3$:	C = 74.53 %	H = 10.56 %	m.w. = 322.
„ „ $C_{19}H_{30}(OCOCH_3)_2$:	C = 73.40 %	H = 9.56 %	m.w. = 376.
„ „ $C_{19}H_{31}O.OCOCH_3$:	C = 75.45 %	H = 10.18 %	m.w. = 334.
„ „ $C_{20}H_{32}(OCOCH_3)_2$:	C = 73.85 %	H = 9.74 %	m.w. = 390.
„ „ $C_{20}H_{33}O.OCOCH_3$:	C = 75.86 %	H = 10.34 %	m.w. = 348.

These results would indicate that the preparation from batch P.U. 4 was almost certainly the pure di-acetate of $C_{19}H_{30}(OH)_2$ or $C_{20}H_{32}(OH)_2$, while that from batch P.U. 5 was a mixture of the mono- and di-acetates. These conclusions probably explain the observations on the melting-points of the two preparations.

Examination of normal urines for the unidentified compound.

Two large batches of urine from normal healthy males have been worked up by the methods previously described.

The first batch of 40 litres was collected from about fifteen different workers in the laboratory, and after thorough extraction with ether and saponification with aqueous alkali yielded 0.146 g. of unsaponifiable matter. This material showed considerable oestrus-producing activity [Fee, Marrian and Parkes, 1929], but apart from traces of a dark red amorphous pigment, it was all readily soluble in a very small volume of ether.

The second batch of 23 litres was collected from twenty-four healthy male students and yielded 0.171 g. of unsaponifiable matter. On treatment with ether none of the alcohol was observed.

21 litres of urine from healthy non-pregnant women were extracted with toluene and ether in the manner described for batches P.U. 4 and P.U. 5, yielding 0.185 g. of unsaponifiable matter. This was all completely soluble in a very small volume of ether.

From these experiments it must be concluded that the alcohol is not present in the urine of healthy males and healthy non-pregnant females.

DISCUSSION.

The yields obtained can in no case be considered to represent the total oestrin content of the urine, or even the maximum obtainable by the methods in general use. In the first place it has been already pointed out that the extraction of the unsaponifiable matter by dilute acetic acid leaves a large amount of the activity in the residue [Marrian and Parkes, 1929] and this doubtless accounts for the low yields for batches S.B. 1 and S.B. 2. Secondly, recent work by Glimm and Wadehn [1929] and by Funk [1929] suggests that the extraction of the hormone from alkaline media by ether may be far from complete. In this case there would be a serious loss of activity on saponification. The object of preparing the extracts, however, was merely to obtain standard preparations so that the stability and methods of further purification of the hormone could be studied quantitatively.

The significance of the dihydroxy-alcohol in the urine of pregnancy is not at present clear. Although it appears to be absent from male urine and from the urine of non-pregnant females, no claim as to its specificity to pregnancy can be made at present. At one time it was thought that it might be a substance, or the metabolic product of a substance, administered to the patients

Although enquiry failed to reveal any such likely substance, this possibility must still be borne in mind.

The frequency of pathological conditions and metabolic disturbances during pregnancy might conceivably be the cause of the appearance of this alcohol in the urine. Until further work has been carried out, however, no conclusions can be drawn.

SUMMARY.

1. Methods of preparing active oestrus-producing concentrates from the urine of pregnant women are described in detail. These methods, while probably giving a low yield, result in preparations suitable for further chemical work on the hormone.

2. There is present in the unsaponifiable fraction of the ether-soluble material from the urine of pregnancy varying amounts of a solid, saturated alcohol. The purification and properties of this alcohol are described. Its molecular formula is considered to be either $C_{19}H_{30}(OH)_2$ or $C_{20}H_{32}(OH)_2$.

3. Although this substance could not be detected in the urine of normal males and normal non-pregnant females, no claim as to its specificity to pregnancy is made.

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