CXV. THE CHEMISTRY OF OESTRIN.
IV. THE CHEMICAL NATURE OF CRYSTALLINE PREPARATIONS.

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(*Received July 1st, 1930.*)

Introduction.

During the last few months several claims have been made to have isolated the oestrus-producing hormone in a crystalline form. Owing to the different methods of assay employed by different workers, it is difficult to make a comparison of these preparations on the basis of their physiological activity. In the opinion of the author differences in the technique of biological assay may result in apparent variations in potency of several hundred per cent in a single preparation. In order to decide therefore the identity or otherwise of these preparations, chemical and physical data must be relied on.

Wieland, Straub and Dorfmüller [1929] described the preparation of active crystalline material melting at 154–165°, finally becoming liquid at 210°. As the authors themselves suggest, the material was probably not a single chemical substance. The activity of the preparation tested in aqueous solution by giving five injections over a period of about 48 hours was as low as 2,000,000 mouse units per g. This is probably significantly lower than the potency of some more recent preparations.

Butenandt [1929] prepared an active crystalline substance, which from the constancy of its activity after recrystallisation and resublimation he believed to be the hormone itself. The substance melted at 240° with decomposition, and molecular weight determinations and analyses pointed to the molecular formula $C_{23}H_{28}O_3$ or $C_{24}H_{32}O_3$. It was found to be unsaturated. On account of its behaviour with aqueous alkali, he suggested that the substance was a hydroxylactone. In a later paper [Butenandt and Ziegner, 1930] the melting point is given as 243°.

Shortly afterwards Dingemanse *et al.* [1930] described what appeared to be the same substance, judging from the melting point and analytical figures. There was some difference in the apparent activity of the two preparations, but this may possibly be accounted for by the fact that Butenandt injected an oily solution of the hormone [Butenandt and Ziegner, 1930]. This point has been discussed in a previous paper [Marrian, 1930]. Dingemanse *et al.* are not yet satisfied that their substance is the pure hormone, since several of their preparations had a significantly higher potency than others.

Doisy, Veler and Thayer [1930] have recently prepared what they claim to be the hormone itself in crystalline form. The crystals showed a constant activity and melting point after as many as twenty recrystallisations from several different solvents. In a later paper [Thayer, Veler and Doisy, 1930], evidence that the substance has the molecular formula $C_{18}H_{21}(OH)_2$ is put forward[1]. Iodine value determinations showed that one double bond was present. The melting point (243° uncorr., 249° corr.) suggests that this substance may be identical with those of Butenandt, and of Dingemanse *et al.* The published combustion figures for these two latter preparations do not differ widely from those required for $C_{18}H_{23}O_2$. It seems possible, therefore, that Butenandt may have been wrong in suggesting that his substance was a hydroxylactone of the formula $C_{23}H_{28}O_3$ or $C_{24}H_{32}O_3$.

In a further communication [Veler, Thayer and Doisy, 1930], they suggest, as other workers have previously done, that the hormone is weakly acidic, and they describe their method of isolation in greater detail.

In a previous paper in this series [Marrian, 1930] the isolation of an active crystalline substance was described. As far as could be judged by a microscopical examination of the crystals, it seemed to consist of a single chemical substance. Preliminary analyses and molecular weight determinations suggested the formula $C_{18}H_{24}O_3$. Since, however, the melting point was indistinct although considerably higher than that of any other preparation (256–261°), and since the crystals were slightly pigmented, no great confidence was placed in this formula. The smallness of the amount of material available at the time made further purification impracticable.

A further batch of the active crystalline substance has since been prepared by the same methods. By using ethyl acetate for the final recrystallisation instead of aqueous methyl alcohol, the crystals were obtained in what was obviously a much purer state. The melting point was higher and sharper than that of the original preparation (264–266° uncorr.), while analyses and molecular weight determinations confirmed the originally suggested molecular formula $C_{18}H_{24}O_3$.

The substance had weak but quite definite acidic properties. It was slowly but completely soluble in cold aqueous alkali and was precipitated from such a solution by carbon dioxide. It was quite insoluble in aqueous Na_2CO_3 solutions.

By treatment with hot acetic anhydride a crystalline acetate was obtained. From the weight of the crude acetate, analyses and molecular weight determinations, it was evident that three hydroxyl groups had been acetylated. Since this accounted for all the oxygen present, the acidic properties of the substance were evidently due to one or more phenolic hydroxyl groups. The acetate itself was quite insoluble in cold aqueous alkali, thus confirming the conclusion that the acidic group or groups had been acetylated.

[1] This is the formula put forward by Thayer, Veler and Doisy, but their results would agree equally well with the more probable formulae $C_{18}H_{20}(OH)_2$ or $C_{18}H_{22}(OH)_2$.

A hydrogen electrode titration showed that one molecule of the substance combined with only one equivalent of base. Only one acidic hydroxyl group was therefore present.

The possible relationship between this substance and other crystalline preparations is discussed in a later section of this paper.

Experimental.

Isolation of the active substance.

The methods of extraction of the hormone from the urine and subsequent methods of purification previously described [Marrian, 1930] were closely followed.

245 litres of urine were acidified and extracted with ether. The ether extract contained 4,600,000 mouse units, corresponding to a yield of 18,800 units per litre. The dried extract was heated with aqueous KOH, and carbon dioxide passed into the mixture for 12 hours, which was then extracted thoroughly with ether. The ether extract was evaporated to dryness and extracted with ice-cold acetone. The acetone extract, containing the hormone, was dried and extracted with ice-cold 50 % alcohol. The alcoholic extract was evaporated to dryness, dissolved in ether after the addition of a small amount of alcohol and extracted with aqueous KOH. After acidification of this alkaline extract, the hormone was extracted with ether.

This concentrate was dissolved in a small amount of alcohol and, after the addition of a large volume of redistilled ether, cooled to − 15°. A large amount of solid material separated and was filtered off. This solid was then boiled in alcoholic solution with charcoal, filtered and evaporated to dryness. On crystallisation of the residue from ethyl acetate, 0·181 g. of white crystalline material was obtained. These crystals melted at 264–266° (uncorr.) with very slight darkening, there being also a slight preliminary shrinkage and darkening at 255°.

Two assays of the potency of this substance gave values of 7,930,000 and 7,460,000 mouse units per g. The total amount of active material in crystalline form was therefore about 1,490,000 mouse units, corresponding to a yield of over 32 % of the activity of the original ether extract.

Solubilities and general properties.

The substance was characterised by a fairly low solubility in most of the common organic fat solvents. In ether the solubility was very low, in methyl and ethyl alcohols, chloroform and acetone it was somewhat greater. It was easily soluble, however, in pyridine.

In 5 % aqueous KOH it dissolved slowly but completely in the cold. On passing carbon dioxide into this solution, it was immediately precipitated. The melting point of this precipitated material was 263–265°, and its physiological activity determined as 7,430,000 and 7,350,000 mouse units per g. by duplicate assays.

In aqueous Na_2CO_3 solution it was quite insoluble.

Determinations of the iodine value by the method of Rosenmund and Kuhnhern using a solution of bromine in pyridine sulphate gave figures which at first suggested the presence of two double bonds. When the phenolic nature of the compound was demonstrated, it was realised that part of the bromine uptake might have been due to bromination of the phenyl radical. By this time insufficient material remained to determine the degree of unsaturation by other methods.

Wieland, Straub and Dorfmüller [1929] observed that their crude crystalline preparation gave an orange colour with a green fluorescence with the Liebermann-Burchardt reaction. A similar colour reaction was noted by the author [Marrian, 1930].

It has now been found that the crystalline preparation behaves in the same way merely on warming with concentrated H_2SO_4. The presence of chloroform and acetic anhydride seems to be unnecessary. The reaction was shown strongly by 0·02 mg. The colour given is indistinguishable from that given by crude bile acids under the same conditions.

Lipschutz's so-called "oxycholesterol" reaction with benzoyl peroxide, acetic and sulphuric acids gave a delicate rose-pink colour with 0·02 mg. of the substance.

Since the substance is probably unsaturated, Rosenheim's [1929] trichloroacetic acid test was tried. It was entirely negative on about 0·5 mg.

Optical rotation.

The rotation was determined in pyridine solution in a Fischer micro-tube using the mercury green line. In order to minimise personal errors, readings were taken by two independent observers.

8·1 mg. of the crystalline substance were dissolved in 0·3 cc. of dry, redistilled pyridine.

	C. R. H.	G. F. M.
Observed rotation =	+ 0·51°	+ 0·52°
$[\alpha]_{5461}$ =	+ 37·7°	+ 38·5°.

Analyses and molecular weight.

Micro-combustions and molecular weight determinations by Rast's camphor method gave the following results:

4·613 mg. gave 12·655 mg. CO_2 and 3·51 mg. H_2O.

C = 74·82 %, H = 8·51 %.

4·437 mg. gave 12·145 mg. CO_2, and 3·29 mg. H_2O.

C = 74·67 %, H = 8·30 %.

3·242 mg. gave 8·890 mg. CO_2, and 2·42 mg. H_2O.

C = 74·79 %, H = 8·36 %.

Mol. w. = 286; 268 (Rast.).

Calculated for $C_{18}H_{24}O_3$:

C = 75·00 %, H = 8·33 %.

Mol. wt. = 288.

These results can leave little doubt that the substance examined is represented by the molecular formula $C_{18}H_{24}O_3$.

Preparation and properties of the acetate.

The general solubilities of the substance suggested the presence of hydroxyl groups. An attempt was therefore made to prepare an acetate. 21·2 mg. were heated to 110° for 2 hours with 2 cc. acetic anhydride. Excess of water was added and after scratching with a glass rod for some time a white solid separated. This was filtered off and was washed repeatedly with water. The crude acetate was then dissolved in hot alcohol and the alcoholic solution evaporated to dryness in a vacuum desiccator over solid KOH. The residue, which was a clear gum, was twice redissolved in alcohol and evaporated over KOH to ensure the complete removal of acetic anhydride.

32·2 mg. of crude acetate were obtained. If three hydroxyl groups had been acetylated, the theoretical weight of acetate should be 30·4 mg.

This crude acetate was dissolved in 1 cc. of hot methyl alcohol and hot water added drop by drop until a faint permanent cloudiness was produced. After cooling for some hours at 0°, rosettes of white needles were deposited. The crystals were filtered off, dissolved in a small volume of alcohol and reprecipitated by the addition of a large volume of water.

The melting point of this preparation was 117·5–119°. A later preparation melted at 120–122°.

Unlike the parent substance, the acetate was quite insoluble in aqueous KOH.

The physiological activity of a specimen of acetate (M.P. 120–122°) was 3,740,000 mouse units per g. On the basis of the amount of original substance present in the acetate, the activity corresponds to 5,370,000 mouse units per g. Thus the conversion of the substance to its acetate appeared to have decreased its physiological activity to a significant extent.

Analysis and molecular weight of the acetate (M.P. 117·5–119°).

Analyses and molecular weight determinations clearly confirmed the view that three hydroxyl groups had been acetylated:

4·965 mg. gave 12·625 mg. CO_2 and 3·23 mg. H_2O.

C = 69·35 %, H = 7·28 %.

4·699 mg. gave 11·950 mg. CO_2 and 3·09 mg. H_2O.

C = 69·35 %, H = 7·36 %.

Mol. wt. = 377; 402 (Rast).

Calculated for:

$C_{18}H_{23}O_2 . OCOCH_3$,	C = 72·73 %,	H = 7·88 %,	mol. wt. = 330.
$C_{18}H_{22}O . (OCOCH_3)_2$,	C = 70·96 %,	H = 7·53 %,	mol. wt. = 372.
$C_{18}H_{21} . (OCOCH_3)_3$,	C = 69·56 %,	H = 7·25 %,	mol. wt. = 414.

Hydrolysis of the acetate.

0·0315 g. of acetate (M.P. 120–122°) was heated for 1 hour to 100° with alcoholic sodium ethoxide in an atmosphere of nitrogen. Excess of water was added and the mixture saturated with carbon dioxide, when a white solid separated. This was filtered off, washed repeatedly with water, dried and recrystallised from ethyl acetate. This substance melted at 267–268° with decomposition. Its physiological activity was found to be 7,400,000 mouse units per g. It seems certain therefore that saponification of the acetate yielded the original substance again.

Hydrogen electrode titration of the active substance.

The data presented in the preceding sections show clearly that the active compound is a trihydroxy-substance and that the acidic properties are due to the presence of one or more acidic hydroxyl groups. It was obviously impossible to determine the number of the acidic groups by direct titration. Mr Kekwick and Mr Cannan therefore very kindly undertook to carry out a hydrogen electrode titration.

The electrode technique was that described by Cannan and Knight [1927]. 20 cc. of a 0·01 *M* solution of the active compound in 0·1 *M* NaOH were titrated with 0·1 *M* HCl. The solution remained free from precipitate throughout the greater part of the titration during which the p_H range covered was 12·7–11·5. Calculation showed that the base bound by the substance within this range was constant and corresponded to an equivalent weight of 268–298. At p_H 11·5 precipitation commenced and simultaneously the amount of base bound to the substance progressively diminished, the curve indicating the presence of a group influencing ionisation within the p_H range 9–11·5. The curve was, however, distorted by the presence of the solid phase. It was assumed that this was due to the low solubility of the undissociated molecule.

A rough determination of the solubility of the substance in 0·1 *M* NaCl was made physiologically. A small quantity of the crystalline substance was shaken for 3 days with 0·1 *M* NaCl at the temperature of the experiment. The solid was allowed to settle and a small volume of the supernatant fluid pipetted off. The physiological activity of this was then determined in the usual manner. In this way it was shown that the solubility of the substance in 0·1 *M* NaCl was of the order of 0·002–0·003 %, *i.e.* approximately 0·0001 *M*.

Assuming that this value applied under the conditions of the experiment, appropriate corrections of the experimental titration curve were made. As a result a p_K of approximately 10·8 was deduced. Making allowances for the

uncertainties involved in the assumptions made, it may be stated that the p_K value is probably within the range 10·5–11·0. It may therefore be said with some certainty that one and one only of the three hydroxyl groups in the molecule is acidic.

Phenolic nature of the acidic hydroxyl group.

The magnitude of the approximate p_K is of the order of that of a phenolic group. However, further proof of the phenolic character of the substance was sought. Certain colour reactions characteristic of phenolic substances were therefore carried out on a colloidal aqueous suspension of the substance, made by adding water to an alcoholic solution.

Millon's reaction was strongly positive. With diazotised *p*-nitraniline[1] a stable purple colour resulted, which was indistinguishable from that given by tyrosine under the same conditions.

On warming some of the dry substance with strong HNO_3 (xanthoproteic reaction), the solid immediately turned a canary yellow, subsequently dissolving to form a yellow solution.

These reactions definitely show that a hydroxyphenyl radical is present in the molecule.

Attempted distillation of the active substance.

The preparations of Butenandt [1929] and of Dingemanse *et al.* [1930] apparently distilled under reduced pressure with some ease. The former preparation distilled at 130–150° and 0·02–0·03 mm. Hg, the latter at 130–150° and 0·01 mm. It was therefore of interest to see whether the compound under investigation would behave in the same way. The author wishes to express his gratitude to Mr Askew, who kindly carried out the distillation at the National Institute for Medical Research.

The experiment was performed on 20 mg. of the crystalline substance, representing about 154,000 mouse units, at a pressure of approximately 0·001 mm. Hg.

The temperature was first raised to 146° for about 45 minutes. Since there was no visible distillate, the temperature was raised to 156° for 1 hour. At this temperature a small amount of material sublimed in the cooler part of the distillation tube, but the process was evidently so slow that the temperature was further raised to 165° for 1 hour. Only very slightly more material sublimed at this temperature. Since there appeared to be a slight degree of decomposition at this temperature, the experiment was abandoned.

The amount of material sublimed was so small that it was quite impossible to weigh it accurately or to determine its physiological activity. Since it melted at 260° it seemed that the original substance had sublimed unchanged.

The unsublimed residue weighed 19·6 mg. and melted at 261–263°. Its physiological activity was unchanged (8,330,000 mouse units per g.).

[1] Unpublished work by Harington and Shüpbach.

Discussion.

The outstanding problem is to decide the relationship between the active trihydroxy-substance described in this paper and the active dihydroxy-substance isolated by Thayer, Veler and Doisy, which may or may not be identical with that isolated by Butenandt.

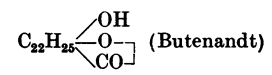
A possible explanation would be to assume that the trihydroxy-substance owes its activity to contamination with the substance melting at 243°. Putting the relative potency of the latter at the highest possible figure, it would then be necessary to suppose that the former was contaminated with at least 15–20 % of the latter. If this were the case it is difficult to explain the melting point of 264–266° of the author's substance.

The failure of the active compound to distil under conditions even more favourable than those in Butenandt's or Dingemanse's experiments makes it still more improbable that the volatile substance melting at 243° is present as an impurity.

On the other hand the constancy of the melting points of the substances described by these authors after frequent recrystallisations makes it impossible to believe that their substances are mixtures.

It is a curious fact that the acetate prepared by Thayer, Veler and Doisy has roughly the same melting point as that prepared by the author. The similarity may be a coincidence, but nevertheless, it is unfortunate that the former do not appear to have attempted to regenerate the original substance from it by saponification.

The only striking difference between the methods of isolation described in this and previous papers and those adopted by other authors, is that in the former, the extract is treated for some time with hot alkali. It is just possible that such treatment might result in a chemical change without seriously impairing the physiological activity. However, it is difficult to see how a substance of the formula

 (Butenandt)

or $C_{18}H_{21}(OH)_2$ (Thayer, Veler and Doisy) could be changed in this way to a substance of the formula $C_{18}H_{21}(OH)_3$.

Alternatively, it could be supposed that two or even three substances originally present in the urine possess oestrus-producing activity. It seems unlikely, however, that oestrogenic activity could be an intrinsic property of three different molecules. A more probable explanation would be that none of these substances is the hormone itself, but rather that they are different inactive compounds, each associated with minute amounts of the real active principle. It is not at all inconceivable that the different methods of purification should result in the isolation of different substances.

The evidence that any of these substances is the pure hormone cannot yet be accepted as conclusive. The fact that the activity is the same for different

batches and is unchanged after recrystallisations is not definite proof. It will be recalled that frequent recrystallisations were found by many workers to be quite ineffective for the removal of provitamin D from cholesterol or phytosterol. The association of oestrogenic activity with the substances under discussion may possibly be found to be a similar problem.

On the other hand, it is curious that the author has so far failed to isolate any substance from urine with a melting point of 243°, while the other authors have not so far reported the isolation of the higher melting substance.

A fact which may be of some significance is that the substances isolated by Dingemanse *et al.*, Doisy, Veler and Thayer and by the author are all stated to exist in two crystalline forms—plates or leaflets, and needles.

It must be admitted that at present no explanation can be put forward to explain all the facts adequately.

Summary.

1. Of the total oestrus-producing material in the ether extract of acidified urine 32 % has been obtained as a pure crystalline substance.

2. This substance (M.P. 264–266°) is represented by the molecular formula $C_{18}H_{24}O_3$. It is strongly dextro-rotatory. $[\alpha]_{5461} = + 38°$.

3. The substance is weakly acidic, being soluble in aqueous alkali. It is precipitated from alkaline solution by carbon dioxide.

4. An acetate (M.P. 120–122°) has been prepared. Analyses and molecular weight determinations show that three hydroxyl groups are acetylated. The acetate is insoluble in aqueous alkali.

5. Determination of the base-binding power and approximate p_K of the substance show that one hydroxyl group is acidic.

6. Certain colour reactions characteristic of phenolic substances are strongly positive. It is concluded that a hydroxyphenyl radical is present in the molecule.

7. The activities and melting-points of the material precipitated from alkaline solution by carbon dioxide and of the material obtained by saponification of the acetate are the same as those of the original substance.

8. The substance is not appreciably volatile at 165° and 0·001 mm. Hg.

9. The relationship between this active substance and the active substances described by other authors is discussed.

The author is again indebted to Prof. F. J. Browne of University College Hospital for providing facilities for the collection of urine. Sincere thanks are due to Prof. J. C. Drummond and to Dr C. R. Harington for their keen interest and frequent helpful advice.

The expenses of this work were defrayed by a grant from the Medical Research Council to whom the author wishes to express his gratitude.

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