III. THE CHEMISTRY OF OESTRIN.

V. THE MECHANISM OF THE CONVERSION OF TRIHYDROXYOESTRIN INTO KETOHYDROXYOESTRIN.

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(Received November 17th, 1931.)

IN previous papers [Marrian 1930, 1, 2, 3] the isolation from the urine of pregnant women of an oestrus-producing crystalline substance characterised as a trihydroxy-compound of the formula $C_{18}H_{21}(OH)_3$ was described. Shortly before this Doisy *et al.* [1929], Butenandt [1929] and Dingemans *et al.* [1930] had reported the isolation of an active crystalline substance from the same source, which from the melting-point and from the analytical data was clearly different from the compound referred to above. Butenandt [1930, 2] has satisfactorily shown that the second compound is a hydroxyketone of the formula $C_{18}H_{22}O_2$. Thayer, Veler and Doisy [1930] at about the same time suggested the formula $C_{18}H_{21}(OH)_2$ for this substance, but later [Thayer, Levin and Doisy, 1931, 2] accepted Butenandt's view that the substance was a hydroxyketone.

The suggestion is now made that, for the sake of convenience, the names *trihydroxyoestrin* and *keto-hydroxyoestrin* should be provisionally adopted to designate these two active compounds. These names will therefore be used throughout this communication. The adoption of this system of nomenclature seems in many respects preferable to the use of the terms *theelol* and *theelin* that have been suggested by Doisy and his colleagues.

The suggestion was tentatively made [Marrian, 1930, 3] that keto-hydroxy-oestrin might be converted into trihydroxyoestrin by the uptake of one molecule of water at some stage during the process of purification of the crude urine extracts. It was however shown by Butenandt [1931, 1, 2] that both compounds existed together in urine and that keto-hydroxyoestrin could be isolated from the mother-liquors of trihydroxyoestrin when the latter was prepared by the method described by one of the authors [Marrian, 1930, 3]. Doisy and Thayer [1931] have since also isolated trihydroxyoestrin as well as keto-hydroxyoestrin from urine.

Butenandt [1931, 1, 2] has discovered the remarkable fact that, on sublimation in a high vacuum with potassium bisulphate, trihydroxyoestrin may be converted with loss of the elements of water into ketohydroxyoestrin identical with that isolated directly from urine extracts.

It was shown [Marrian, 1930, 2, 3] that one of the hydroxyl groups in trihydroxyoestrin was weakly acidic in character. Since the substance also gave colour reactions characteristic of phenols it was inferred that this acidic hydroxyl was phenolic. Similar views have since been expressed by Thayer, Levin and Doisy [1931, 1]. Ketohydroxyoestrin is also a weakly acidic substance, as Butenandt [1929] originally showed. Butenandt [1931, 1] suggested that the acidity was due to enolisation of the carbonyl group. Since the elimination of water from trihydroxyoestrin to yield ketohydroxyoestrin must involve the intermediate formation of such an enolic form, this suggestion appeared feasible. On the other hand, Thayer, Levin and Doisy [1931, 2] have suggested that the acidity is due to "acidic ionisation of the hydroxyl." Their evidence for this view is that ketohydroxyoestrin on methylation yields a monomethyl ether which is insoluble in alkali and which does not give a positive Millon reaction. They appear, however, to have overlooked the possibility that the group which can be methylated and the one responsible for the positive Millon reaction, might conceivably be the enolised carbonyl group postulated by Butenandt.

The work described in this paper was designed to settle definitely whether the acidity of ketohydroxyoestrin is due to the phenolic nature of the hydroxyl group, or whether it is due to enolisation of the carbonyl group. If the former view is correct, then in the conversion of trihydroxyoestrin to ketohydroxyoestrin, water must be eliminated from the two non-acidic hydroxyls. If the latter view is correct, then the phenolic hydroxyl in trihydroxyoestrin must be involved in the elimination of water to form dioxyoestrin. It is clearly not possible that the acidity of ketohydroxyoestrin is due to a phenolic hydroxyl and also to an enolised carbonyl group, since the monomethyl ether of ketohydroxyoestrin is insoluble in alkali.

The problem was easily and conclusively settled in the following manner. The monomethyl ether of trihydroxyoestrin was sublimed in a high vacuum with potassium bisulphate in the manner described by Butenandt for the conversion of trihydroxyoestrin into ketohydroxyoestrin. The product so obtained proved to be identical in melting-point, optical rotation, and crystalline form with the monomethyl ether of ketohydroxyoestrin. Clearly, therefore, water was eliminated from the two unmethylated alcoholic hydroxyl groups. As a further proof, it has been shown that ketohydroxyoestrin methyl ether and the dehydration product of trihydroxyoestrin methyl ether give identical oximes.

EXPERIMENTAL.

Methods.

Physiological tests of the potency of the compounds were carried out in the manner described in previous papers [Marrian, 1930, 1, 3].

Optical rotations were determined in a 0.5 dm. Fischer micro-tube, using the mercury-green line. Since the possible errors in such micro-determinations are considerable, the determinations have been done in duplicate where possible.

The melting-points recorded are uncorrected. In all cases they were carried out with short-stemmed Anschütz thermometers.

Micro-analyses were carried out by Dr A. Schoeller of Berlin. In all cases the substances were dried at 80° over phosphorus pentoxide in a high vacuum before combustion.

Preparation and purification of trihydroxyoestrin.

The trihydroxyoestrin used in this work was an accumulation from different batches worked up by different methods. The method of isolation described here is the one found by experience to yield the best results and incorporates several of the purification processes first described by Butenandt.

The urine was acidified with sulphuric acid and extracted four to six times with ether. The ethereal extract, after washing with water until free from acid, was evaporated and heated to 100° with 5 % aqueous potassium hydroxide for 2 hours. The alkaline mixture after saturation with carbon dioxide was thoroughly extracted with ether. The residue from the evaporation of this extract was boiled with dilute alcoholic hydrochloric acid [Butenandt, 1930, 1]. This acid mixture, after dilution with water, was extracted with ether, and the ethereal extract was washed with aqueous sodium carbonate solution several times. Phenolic substances were then removed from this ethereal extract by repeatedly shaking with aqueous potassium hydroxide. The aqueous alkaline solution was extracted with ether after saturation with carbon dioxide. It was found to be advantageous to extract this ethereal solution once more with alkali, since otherwise small quantities of non-phenolic unsaponifiable substances occasionally contaminated the final product. The final ethereal extract, after thorough washing with water, was evaporated to dryness and the residue was dissolved in 60 % aqueous ethyl alcohol. This solution was then extracted several times with benzene to remove the fraction containing the ketohydroxyoestrin. The benzene solution was washed with 50 % aqueous methyl alcohol, these washings being added to the main 60 % ethyl alcohol fraction [cf. Butenandt, 1931, 1]. The total aqueous alcoholic fraction after partial evaporation of the alcohol and dilution with water was extracted with ether, and the ethereal extract evaporated to dryness.

Crude trihydroxyoestrin was separated from this by dissolving in a small

volume of ethyl acetate and keeping at 0° for 24–48 hours. This crude material was cleaned by boiling in alcoholic solution with charcoal. The residue, after the evaporation of the alcoholic filtrate, was washed with a small volume of ice-cold ethyl acetate and dried. The product, when prepared in this way, had a melting-point usually between 276 and 280° and a physiological potency of 7–9 million mouse units per g.

The combined batches of crystalline material, after a second treatment with charcoal in alcoholic solution, were dissolved in *N* sodium hydroxide and filtered from small amounts of insoluble flocculent material. The alkaline filtrate was saturated with carbon dioxide and the precipitated trihydroxy-oestrin was filtered off, washed and dried. The product was finally recrystallised from aqueous ethyl alcohol; m.p. 279·5–280·5° (slight decomp.).

4·930 mg. gave 13·550 mg. CO₂, 3·67 mg. H₂O,

C = 74·95 %, H = 8·27 %.

Calculated for C₁₈H₂₄O₃, C = 75·00 %, H = 8·33 %.

Optical rotation: $c = 2·58$ in pyridine, $\alpha = +0·554^\circ$, $[\alpha]_{5461}^{22} = +42·9^\circ$,

$c = 3·35$ in pyridine, $\alpha = +0·687^\circ$, $[\alpha]_{5461}^{23} = +40·8^\circ$,

$c = 0·432$ in alcohol, $\alpha = +0·159^\circ$, $[\alpha]_{5461}^{21} = +78·3^\circ$,

$c = 0·261$ in alcohol, $\alpha = +0·096^\circ$, $[\alpha]_{5461}^{22} = +73·6^\circ$.

Physiological potency: 7,630,000 m.u. per g.

Conversion of trihydroxyoestrin into ketohydroxyoestrin.

0·66 g. trihydroxyoestrin was mixed intimately with 16 g. of powdered fused potassium bisulphate and placed in a small hard glass retort of about 20 cc. capacity. The retort was heated in an air-bath for 5 hours at 110°, 3 hours at 140°, and finally 6 hours at 150°, at 0·02–0·05 mm. The contents of the whole retort were then washed out and suspended in a large volume of *N*/100 sodium hydroxide. The ketohydroxyoestrin was obtained from this mixture by repeated extraction with ether [*cf.* Veler, Thayer and Doisy, 1930]. The residue left on evaporation of the ethereal extract was washed twice with small volumes of ice-cold ether to remove traces of pigmented impurities, boiled in alcoholic solution with charcoal, and finally recrystallised from aqueous methyl alcohol. 0·22 g. of material melting with decomposition at 245–252° was obtained. The material was purified by sublimation for 20 hours at 150–160° and 0·01–0·02 mm. The final product melted at 242–246° with decomposition.

5·220 mg. gave 15·075 mg. CO₂, 3·880 mg. H₂O,

C = 78·75 %, H = 8·26 %.

Calculated for C₁₈H₂₂O₂, C = 80·00 %, H = 8·15 %.

Optical rotation: $c = 0·2675$ in ethyl alcohol, $\alpha = +0·201^\circ$, $[\alpha]_{5461}^{19} = +188^\circ$.

Physiological potency: 16,300,000 and 19,400,000 m.u. per g.

Preparation of trihydroxyoestrin methyl ether.

0.261 g. of trihydroxyoestrin was dissolved in 50 cc. *N*/2 sodium hydroxide and shaken for 2 hours with 2.5 cc. of dimethyl sulphate. The product was filtered off, suspended in 20 cc. *N*/2 sodium hydroxide, and warmed for 1 hour to dissolve all traces of unmethylated material. The insoluble residue was filtered off, washed thoroughly with water, and recrystallised once from aqueous ethyl alcohol and finally from absolute ethyl alcohol; M.P. 162.5–164°.

4.790 mg. gave 12.965 mg. CO₂, 3.77 mg. H₂O,

C = 73.82 %, H = 8.74 %.

3.080 mg. gave 2.355 mg. AgI (micro-Zeisel),

(CH₃O –) = 10.09 %.

Calculated for C₁₈H₂₃O₂.OCH₃, C = 74.44 %, H = 8.86%, (CH₃O –) = 10.26 %.

Optical rotation: *c* = 0.404 in ethyl alcohol, α = + 0.131°, [α]₅₄₆₁¹⁹ = + 64.8°,

c = 0.298 in ethyl alcohol, α = + 0.098°, [α]₅₄₆₁²⁰ = + 65.8°,

c = 0.481 in pyridine, α = + 0.072°, [α]₅₄₆₁²⁰ = + 29.9°.

Preparation of ketohydroxyoestrin methyl ether.

0.030 g. of ketohydroxyoestrin was dissolved with warming in 20 cc. *N*/2 sodium hydroxide and shaken for 2 hours with 0.5 cc. dimethyl sulphate. The product was filtered, and treated with a further quantity of *N*/2 alkali, as in the previous preparation. The washed residue, insoluble in alkali, was dried and recrystallised from absolute methyl alcohol after purification with charcoal in ethyl alcohol; M.P. 164–167°.

4.193 mg. gave 12.245 mg. CO₂, 3.200 mg. H₂O,

C = 80.05 %, H = 8.48 %.

3.066 mg. gave 2.650 mg. AgI (micro-Zeisel),

(CH₃O –) = 11.40 %.

Calculated for C₁₈H₂₁O.OCH₃, C = 80.28 %, H = 8.45 %, (CH₃O –) = 10.91%.

Optical rotation: *c* = 0.330 in chloroform, α = + 0.283°, [α]₅₄₆₁²¹ = + 171°,

c = 0.440 in chloroform, α = + 0.377°, [α]₅₄₆₁²⁰ = + 171°.

Dehydration of trihydroxyoestrin methyl ether.

0.104 g. trihydroxyoestrin methyl ether was intimately mixed with 2.5 g. of powdered fused potassium bisulphate and heated in a retort for 2 hours at 90° and 19 hours at 110–120° at 0.01 mm. The product which sublimed in the neck of the retort weighed 0.041 g. It was recrystallised twice from aqueous ethyl alcohol and finally from absolute methyl alcohol.

Optical rotation: *c* = 0.552 in chloroform, α = + 0.446°, [α]₅₄₆₁²⁰ = + 171°,

c = 0.426 in chloroform, α = + 0.374°, [α]₅₄₆₁²⁰ = + 176°.

It had m.p. 163–167°; mixed melting-point with ketohydroxyoestrin methyl ether, 162–168.5°. Crystals from absolute ethyl alcohol were similar in form to those of ketohydroxyoestrin methyl ether from the same solvent.

Preparation of the oxime of ketohydroxyoestrin methyl ether.

Ketohydroxyoestrin methyl ether was dissolved in ethyl alcohol and warmed for 2 hours with hydroxylamine hydrochloride and excess of sodium acetate. Excess of water was added and the precipitated oxime filtered off. The crude oxime was redissolved in alcohol and again precipitated with water; m.p. 182–187°.

A small amount of the trihydroxyoestrin methyl ether dehydration product was oximated in the same manner as described above; m.p. 183–187°; mixed melting-point with ketohydroxyoestrin methyl ether oxime, 180–184°.

SUMMARY.

1. Starting with trihydroxyoestrin ($C_{18}H_{24}O_3$; m.p. 279.5–280.5°; $[\alpha]_{5461} + 41.9^\circ$ in pyridine, $+ 76^\circ$ in ethyl alcohol; 7.6 million m.u. per g.) the following compounds have been prepared.

(a) *Ketohydroxyoestrin* ($C_{18}H_{22}O_2$; m.p. 242–246°; $[\alpha]_{5461} + 188^\circ$ in ethyl alcohol; 17.8 million m.u. per g.) by distillation of trihydroxyoestrin with potassium bisulphate in a high vacuum.

(b) *Ketohydroxyoestrin monomethyl ether* (m.p. 164–167°; $[\alpha]_{5461} + 171^\circ$ in chloroform) by treatment of ketohydroxyoestrin with dimethyl sulphate in alkaline solution.

(c) *Trihydroxyoestrin monomethyl ether* (m.p. 162.5–164°; $[\alpha]_{5461} + 29.9^\circ$ in pyridine, $+ 65.3^\circ$ in ethyl alcohol) by treatment of trihydroxyoestrin with dimethyl sulphate in alkaline solution.

(d) *Dehydration product of trihydroxyoestrin methyl ether* (m.p. 163–167°; $[\alpha]_{5461} + 173^\circ$ in chloroform) by distillation of trihydroxyoestrin methyl ether with potassium bisulphate in a high vacuum.

(e) *Oxime of ketohydroxyoestrin methyl ether* (m.p. 182–187°).

(f) *Oxime of dehydration product of trihydroxyoestrin methyl ether* (m.p. 183–187°).

2. Compounds (b) and (d) had similar crystalline forms, the same melting-point, a mixed melting-point showing no depression and the same optical rotation. They are therefore identical.

Compounds (e) and (f) had the same melting-point and gave a mixed melting-point showing practically no depression. They are, therefore, identical.

3. These data are considered to provide conclusive evidence that in the conversion of trihydroxyoestrin to ketohydroxyoestrin water is eliminated between the two non-acidic hydroxyls, and that the acidity of the latter compound is due to a phenolic hydroxyl and not to enolisation of the carbonyl group.

The expenses of this work were defrayed by a special grant from the Medical Research Council, to whom the authors wish to express their thanks.

The authors are also indebted to the various hospital authorities who so kindly provided facilities for the collection of urine.

The extraction of part of the urine was carried out by the British Drug Houses, Ltd.

REFERENCES.

- Butenandt (1929). *Deutsch. med. Woch.* **55**, 2171.
— (1930, 1). *Z. physiol. Chem.* **191**, 127.
— (1930, 2). *Z. physiol. Chem.* **191**, 140.
— (1931, 1). *Abhandl. Ges. Wiss. Göttingen, Math.-phys. Kl.* iii folge, Heft 2.
— (1931, 2). *Z. physiol. Chem.* **199**, 243.
Dingemanse, de Jongh, Kober and Laqueur (1930). *Deutsch. med. Woch.* **56**, 301.
Doisy and Thayer (1931). *J. Biol. Chem.* **91**, 641.
— Veler and Thayer (1929). *Amer. J. Physiol.* **90**, 329.
Marrian (1930, 1). *Biochem. J.* **24**, 435.
— (1930, 2). *Chem. Ind.* **49**, 515.
— (1930, 3). *Biochem. J.* **24**, 1021.
Thayer, Levin and Doisy (1931, 1). *J. Biol. Chem.* **91**, 655.
— — (1931, 2). *J. Biol. Chem.* **91**, 791.
— Veler and Doisy (1930). *Proc. Soc. Exp. Biol. Med.* **27**, 735.
Veler, Thayer and Doisy (1930). *J. Biol. Chem.* **87**, 357.

Note added February 1st, 1932. Since this work was completed, a short paper by Butenandt (*Z. angew. Chem.* **44**, 905) has appeared, in which he shows that the acidity of ketohydroxyoestrin is due to the hydroxyl group, since its esters are insoluble in alkali while derivatives involving the carbonyl group retain their solubility in alkali.