CLXXVII. A NEW SATURATED SOLID ALCOHOL FROM THE URINE OF PREGNANT MARES¹.

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THE recent proof of the close chemical relationship between the oestrus-producing hormones and the sterols and bile acids by Butenandt *et al.* [1933] has led to the widespread belief that these hormones may be produced in the animal body by the degradation of some sterol-like precursor. Pregnandiol, which was shown by Butenandt *et al.* [1931] to have a sterol-like structure, might be considered as a possible intermediate in the transformation of a sterol or bile acid into one of the oestrus-producing hormones. The facts that it is not present in human male urine or in the urine of non-pregnant human females [Marrian, 1929], and that so far it has only been found in human pregnancy urine together with oestrone and oestriol, lend some support to this view. With the object of providing further support for this theory, a careful examination of the neutral ether-soluble fraction of pregnant mares' urine for pregnandiol was undertaken.

The chilling of an acetone solution of the neutral ether-soluble fraction from human pregnancy urine invariably results, in the experience of the authors, in the deposition of crystals of crude pregnandiol. A similar treatment of the neutral fraction of pregnant mares' urine led us to conclude that either pregnandiol was entirely absent or else it was present in amounts too small to be detected in this manner. It had been noticed, however, that in the ether extraction of the neutral material from aqueous alkaline solution, a dark semi-crystalline solid tended to separate out at the ether-water interface. At first hopes were entertained that this might be crude pregnandiol. This material, however, proved to be much more soluble in acetone than pregnandiol. Treatment of the crude solid with charcoal in alcoholic solution, and finally crystallisation from benzene, yielded white crystals which melted with decomposition at about 300° (*cf.* pregnandiol, m.p. 239°). Subsequently it was found that further quantities of this substance could be obtained by chilling a benzene solution of the main neutral fraction.

Superficially, the new compound resembled pregnandiol to some extent. When heated with concentrated sulphuric acid an orange-red colour with a strong green fluorescence was produced. A similar colour is given under the same conditions by pregnandiol, many of the sterols and many of the bile acids. The new compound was more soluble in ethyl alcohol, methyl alcohol and acetone than was pregnandiol, but less soluble in benzene and ether. Iodine

¹ A preliminary account of this work has previously been published [Smith *et al.*, 1933].

value determinations showed that it was fully saturated. These facts, together with the fact of its higher melting-point, suggested the possibility that the new compound might be of the same type as pregnandiol but with a greater oxygen content.

Combustion figures indicated an empirical formula of $(C_7H_{12}O)_n$. Owing to the great insolubility of the compound in molten camphor, it was impossible to determine its molecular weight by Rast's method. Owing to its low solubility in all the usual organic solvents, molecular weight determinations by other methods were also found to be impossible.

Acetylation in the usual manner with acetic anhydride yielded an acetate, the analysis of which showed that all the oxygen in the molecule of the original substance was present as acetyltable hydroxyl groups. The acetate had a much greater solubility in the usual organic solvents and in molten camphor, and therefore it was hoped that it would be possible to obtain reliable figures for its molecular weight. A number of determinations by Rast's method on a specimen of acetate which had been carefully recrystallised, gave values ranging from 377 to 413. These figures are considerably too low for the triacetate of a substance of the formula $C_{21}H_{33}(OH)_3$, but yet are much too high for the diacetate of a substance $C_{14}H_{22}(OH)_2$. The molecular weight would be in close agreement with the diacetate of a substance containing three oxygen atoms. The combustion figures for the acetate did not, however, allow this explanation of the observed facts.

It seemed possible at first that the original alcohol might not be a single substance and that the acetate obtained might be derived from a single component of the mixture. Hydrolysis of the acetate, however, yielded a substance of the same melting-point as the original alcohol, which gave with the latter a mixed melting-point showing no depression.

Since the determined molecular weight of the acetate cannot be reconciled with any of the possible molecular formulae derived from the carbon and hydrogen determination, the former must be considered as an abnormal value. In this connection it might be pointed out that Smith and Young [1927] observed that several pure organic compounds gave abnormal molecular weights by the Rast method.

The molecular dimensions of the new substance are therefore still in doubt. It seems possible that it is a trihydroxy-substance of the formula $C_{21}H_{33}(OH)_3$, perhaps formed by the hydroxylation of pregnandiol, but so far there is no positive evidence in favour of this attractive hypothesis.

So far no single decomposition product of the new compound has been obtained in appreciable amount. Oxidation by chromic anhydride in glacial acetic acid solution under the mildest conditions apparently caused extensive breakdown of the molecule, yielding a mixture of acidic substances from which it was not found possible to isolate a pure compound. It is interesting to contrast the behaviour of pregnandiol with chromic anhydride; the latter is oxidised smoothly and almost quantitatively to a diketone [Butenandt, 1930].

An attempted dehydration by heating to 250–300° at a pressure of about 12 mm. Hg was complicated by the ease with which the substance sublimed under these conditions. Some dehydration had evidently occurred, since from the products there was isolated a small amount of material (m.p. 123°) which had an iodine value of 62. The mother-liquors from the crystallisation of the latter substance yielded on treatment with bromine in ethereal solution small amounts of an insoluble bromide. It was not possible, however, to obtain large enough amounts of these decomposition products for analysis.

Attempts to prepare the parent saturated hydrocarbon by substitution of the hydroxyl groups with halogen and subsequent reduction have not so far been successful.

It seems doubtful if much progress can be made in elucidating the structure of this interesting compound until much larger amounts are available.

EXPERIMENTAL.

Methods of analysis.

Carbon and hydrogen determinations were carried out by Dr Ing. A. Schoeller (Berlin) and by Dr H. Stantial of the Chemistry Department, the University of Toronto. The authors are deeply indebted to Dr Stantial for her willing co-operation.

Molecular weights were determined by Rast's camphor method. Some difficulty was encountered in obtaining a specimen of camphor suitable for the purpose. A specimen of camphor sold specially for the purpose was found to be far from uniform, samples taken from different parts of the bottle varying by as much as 2° in m.p. This material, after crystallisation from alcohol and finally after grinding to a paste with pure ether and allowing to dry in the air, was found to be sufficiently uniform for the determinations. Numerous determinations on substances of known molecular weight and of a high degree of purity gave values close to the theoretical. It may be noted that pregnandiol acetate gave perfectly normal molecular weight values.

The authors are greatly indebted to Miss Dorothy Skill for carrying out these determinations.

All samples were dried over P_2O_5 *in vacuo* at 80° before analysis.

Isolation and purification of the new substance from mares' urine.

The material used in these experiments was a sample of crude extract of the urine of pregnant mares from which the oestrone fraction had previously been separated. It was very kindly placed at our disposal by Dr André Girard of Paris to whom we wish to express our thanks.

The crude extract was heated for 1–2 hours on a boiling water-bath with strong alcoholic potassium hydroxide solution. The mixture was cooled, diluted with about 3 volumes of water and then ether-extracted after the addition of sodium chloride to facilitate the breaking of the troublesome emulsions that formed. The ethereal extract was washed successively with 2*N* sodium hydroxide, 2*N* hydrochloric acid, 2*N* sodium hydroxide and finally twice with water. The gummy residue obtained by the evaporation of this extract to dryness was dissolved in boiling benzene and the solution then placed in the ice-chest. After 1–2 days, a small amount of dark semi-crystalline solid material was deposited. This was filtered off, washed with cold benzene and then dissolved in hot ethyl alcohol and decolorised by boiling with charcoal. The alcoholic filtrate on evaporation to dryness yielded a nearly white crystalline solid. Remaining traces of gummy and pigmented material were removed by washing with cold benzene and by a further treatment with charcoal in alcoholic solution. The final product obtained in this way showed a rather indefinite m.p. of about 295–299° (decomp.) with preliminary darkening and sintering at about 284°. About 5 g. of this material were obtained from 700 g. of the unsaponifiable ether-soluble material of the urine extract.

Purification of this material was effected in the following way. On cooling a solution in methyl alcohol to 0°, a small amount of a white gelatinous material

was precipitated. This was removed by filtration and the filtrate was then concentrated. To this concentrated methyl alcoholic solution was added an excess of benzene, and the mixture was boiled to lower the relative concentration of methyl alcohol until the point of incipient crystallisation was reached. On cooling to 0° and allowing to stand for 24 hours, the substance separated as small white needles.

A sample recrystallised three times in this way melted at 303–304.5° with decomposition and preliminary sintering some degrees lower. The exact melting-point was found to vary considerably over a range of 5° or so according to the rate of heating and according to the tightness with which the specimen was packed into the melting-point tube. It is clear therefore that the exact melting-point is of only limited value as a criterion of purity.

Properties and empirical constitution.

The substance is, like pregnandiol, characterised by an extremely low solubility in most of the common organic solvents. In benzene, light petroleum, hexane and ether the solubility is extremely low, even on boiling. The solubility in ethyl alcohol is rather greater and in methyl alcohol greater still. The best solvents so far found are pyridine and dioxan, but in the cold, solutions of greater strength than 1 % cannot be obtained.

The xanthoproteic reaction was found to be entirely negative, indicating that no aromatic ring is present in the molecule.

The iodine value was determined in the following way. 2.15 mg. were partially dissolved in about 2 ml. of chloroform and then allowed to stand in the dark for 20 minutes in contact with 1.80 ml. of the Rosenmund-Kuhnhenh reagent. Solution was complete immediately after adding the reagent. After the addition of water and 10 % potassium iodide solution, the mixture required 1.875 ml. of *N*/10 thiosulphate to titrate the iodine liberated. A blank experiment took 1.880 ml., showing that the substance was fully saturated.

The optical rotation was determined in pyridine solution in a Fischer 0.5 dm. tube, using the mercury green line. It was not found possible to determine the rotation in methyl alcoholic solution owing to the low solubility of the substance in that solvent. The results are shown in Table I.

Table I. *Optical rotation of new compound (M.P. 303–304.5°).*

Weight in mg.	Pyridine ml.	Observed rotation	Temp. ° C.	$[\alpha]_{5461}$
16.35	1.98	– 0.181°	25	– 43.8°
7.00	0.83	– 0.187	19	– 44.3
8.9	0.90	– 0.219	20	– 44.1

(Found: C, 74.77, 74.59; H, 10.81, 10.85 %. $(C_7H_{12}O)_n$ requires C, 74.92; H, 10.79 %. $C_{20}H_{34}O_3$ requires C, 74.45; H, 10.63 %. $C_{22}H_{38}O_3$ requires C, 75.35; H, 10.93 %.)

Preparation of acetate. 64.4 mg. of the alcohol were heated to 100° for 2 hours with 5 ml. of acetic anhydride and 1 drop of pyridine. After the addition of an excess of water, the insoluble acetate was filtered off, washed thoroughly, dried *in vacuo* over solid calcium chloride and potassium hydroxide and weighed. 88.6 mg. of material of m.p. 164–166° were obtained. The theoretical yield for the complete acetylation of $(C_7H_{11}OH)_n$ would be 88.5 mg. for this amount.

The material was recrystallised twice from methyl alcohol. 45 mg. of m.p. 168° were obtained.

(Found: C, 70.24, 70.28; H, 9.29, 9.16 %. $(C_7H_{11}OCOCH_3)_n$ requires C, 70.08; H, 9.16 %. $C_{20}H_{31}(OCOCH_3)_3$ requires C, 69.60; H, 8.99 %. $C_{22}H_{35}(OCOCH_3)_3$ requires C, 70.51; H, 9.31 %. $C_{21}H_{34}O(OCOCH_3)_2$ requires C, 71.40; H, 9.60 %. $C_{21}H_{35}O_2(OCOCH_3)$ requires C, 72.96; H, 10.13 %. Mol. wt. found: 413, 377, 390, 389, 412, 381; mean 394. $C_{21}H_{33}(OCOCH_3)_3$, $C_{21}H_{34}O(OCOCH_3)_2$ and $C_{21}H_{35}O_2(OCOCH_3)$ require 462, 420 and 378 respectively.)

Hydrolysis of acetate. 18.5 mg. of the acetate were boiled with 5 ml. of 10 % alcoholic potassium hydroxide for 3 hours. Excess of water was then added and the precipitated material was filtered off, washed and dried. After recrystallisation from benzene-methyl alcohol the product melted at 298°, and mixed with the original alcohol melted at 301°.

SUMMARY.

1. From the neutral, ether-soluble fraction of pregnant mares' urine there has been isolated a saturated alcohol, M.P. 303–304.5; $[\alpha]_{5461}^D$, -44° . Analyses are in good agreement with the formula $(C_7H_{12}O)_n$.

2. On acetylation an acetate, M.P. 168°, was obtained which analysed to the formula $(C_7H_{11}O.COCH_3)_n$. Hydrolysis of the acetate yielded the original alcohol.

3. It has not so far been possible to determine satisfactorily the molecular weight of either the alcohol or its acetate.

4. The possibility of the alcohol being closely related to pregnandiol is discussed.

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