CCXV. A NOTE ON THE PRESENCE IN HUMAN PREGNANCY URINE OF AN ACID-HYDROLYSABLE COMBINED FORM OF PREGNANDIOL.

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PREGNANDIOL was isolated from human pregnancy urine by Marrian [1929] and shortly afterwards independently by Dingemanse *et al.* [1930] and by Butenandt [1930]. Its structure was determined by Butenandt *et al.* [1931]. Recently a revived interest has been shown in this compound as a result of the work by Butenandt & Schmidt [1934] who showed that by a simple series of procedures it can be converted into progesterone. In view of the obvious value of pregnandiol as a source of progesterone, it appeared to the authors to be advisable to make a study of the conditions necessary for its complete extraction from urine.

In previous work from this laboratory it has been shown [Cohen *et al.* 1935] that over 99% of the oestrin in human pregnancy urine is in an acid-hydrolysable combined form. More recently it has been shown that the "combined" oestriol may be quantitatively extracted from the urine with butyl alcohol and that part, at least, of this is an oestriol monoglucuronide [Cohen & Marrian, 1936]. It has furthermore been shown [Schachter & Marrian, unpublished] that butyl alcohol will extract most of the "combined" oestrogens present in the urine of pregnant mares. It would also appear probable from the work of Adler [1934] that some of the androgenic material in human male urine is likewise present in a butyl alcohol-soluble, acid-hydrolysable "combined form". In view of these facts it seemed possible that pregnandiol might similarly be excreted in a butyl alcohol-soluble combined form.

EXPERIMENTAL.

The starting material in all these experiments was the butyl alcoholic extract of pregnancy urine which had been washed repeatedly with $N/3$ NaOH to remove the combined oestriol, washed with water and evaporated to dryness. Such a fraction would necessarily contain all the free pregnandiol originally present in the urine and probably any "combined" pregnandiol, provided that the latter was not sufficiently strongly acidic to be removed by the alkali washing.

The general procedure adopted was as follows.

The dry residue from the evaporation of the alkali-washed butyl alcohol was dissolved and suspended in a suitable volume of N NaOH. The ether-soluble neutral substances (including the free pregnandiol) were removed from this mixture by repeated ether extraction. The combined ethereal extracts, after washing with water, were evaporated to dryness and the residue dissolved in the smallest possible volume of hot acetone. The solution after 2-3 days at 0° , deposited crystals of crude pregnandiol. These were filtered off, washed with cold acetone and decolorized by boiling with charcoal in ethyl alcoholic solution. The material obtained by evaporation of the alcoholic filtrate was finally treated with

warm light petroleum in which pregnandiol is nearly insoluble, in order to remove soluble impurities, and weighed. For final identification of this material by its melting point, it was recrystallized from the smallest possible volume of ethyl alcohol.

The original aqueous alkaline phase was then heated on a water-bath to remove all the dissolved ether, adjusted to the desired *pH* and hydrolysed by heating at 100° in a boiling water-bath or at 120° in an autoclave. The hydrolysis mixture was next made alkaline (where necessary) by the addition of NaOH, and repeatedly extracted with ether. Any pregnandiol set free by the hydrolytic treatment was isolated from this ethereal extract by the same procedure as described in the preceding paragraph.

1st experiment. From the butyl alcoholic extract of 100 litres of urine were obtained 46.7 mg. of crude free pregnandiol which after one crystallization from ethyl alcohol melted at 233–234°. After heating the aqueous alkaline phase at 100° for 4 hours only 1.2 mg. more of crude pregnandiol were obtained. The aqueous phase after hydrolysis for 1.5 hours at *pH* 2.0 at 100° yielded only 1.0 mg. more of crude pregnandiol. After a further hydrolysis at *pH* 1.5 at 100° for 2 hours 20 min., 108.2 mg. of crude pregnandiol were obtained, which after one crystallization from ethyl alcohol melted at 230–232°. The mixed m.p. with highly purified pregnandiol (m.p. 236.7–237°) was 230–234°.

2nd experiment. The butyl alcohol-soluble fraction from 360 litres of urine which had previously been extracted with ether was dissolved in NaOH, extracted with ether and then divided into nine equal portions. Each portion was then subjected to a different hydrolytic treatment. The results are shown in the following table.

Hydrolysis	mg. crude pregnandiol
<i>pH</i> 1.5 at room temperature for 8 days	Negligible
<i>pH</i> 1.5 at 100° for 15 min.	3.2
<i>pH</i> 1.5 at 100° for 30 min.	0.3
<i>pH</i> 1.5 at 100° for 60 min.	0.7
<i>pH</i> 1.5 at 100° for 120 min.	36.7
<i>pH</i> 1.5 at 100° for 240 min.	29.0
<i>pH</i> 1.5 at 120° for 30 min.	1.5
<i>pH</i> 1.5 at 120° for 60 min.	46.3
<i>pH</i> 1.5 at 120° for 120 min.	78.4

DISCUSSION.

The experiments described conclusively prove that a considerable fraction of the pregnandiol excreted in human pregnancy urine is in a combined, ether-insoluble form. Since this combined pregnandiol remains in the butyl alcohol after repeated extraction with aqueous *N*/3 NaOH, it is evident that it is a much less strongly acidic compound than is oestriolglucuronide. Since the alkaline washings of the butyl alcoholic extract of pregnancy urine have not yet been examined thoroughly for the presence of combined pregnandiol, the possibility cannot be excluded that other more strongly acidic compounds of pregnandiol are present in this fraction.

A rough determination of the conditions necessary for optimum hydrolysis of this combined pregnandiol has been made. It was not readily hydrolysed by hot *N* NaOH, at *pH* 1.5 in the cold or at *pH* 2.0 at 100° after more than 2 hours. Considerable hydrolysis took place at *pH* 1.5 at 100° after 2 hours, but since the yield after a 4-hour hydrolysis was somewhat lower, it would appear possible that further hydrolysis under these conditions was accompanied by destruction. Hydrolysis in the autoclave at *pH* 1.5 for 2 hours gave considerably higher

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for oestriol monoglucuronide *read* pregnandiol monoglucuronide

yields than were obtained at 100°. It seems possible that the reason for this may be that the destruction of pregnandiol in hot acid solution is oxidative in character, as is the case with oestriol [Cohen & Marrian, 1935], and that in the more anaerobic conditions of the hydrolysis in the autoclave the destruction was decreased.

The attention of the authors was first drawn to the possibility of a destruction of pregnandiol by hot acid by the fact that the melting point of the crude pregnandiol obtained in the acid-hydrolysis experiments usually ranged between 205 and 220°, whereas that of the crude pregnandiol extracted before hydrolysis was usually between 220 and 225°. At first it was thought that the low melting point of the pregnandiol released by hydrolysis might be due to admixture with other substances having similar solubilities in acetone and light petroleum which had also been liberated. It was found, however, that the melting point of pure pregnandiol could be depressed to below 220° by prolonged boiling in aqueous acid solution. Such an explanation was therefore unnecessary.

It must be admitted that these results do not yet provide a wholly satisfactory solution of the practical problem of obtaining larger yields of pregnandiol from pregnancy urine. The conditions of hydrolysis which would be necessary for maximum yields of pregnandiol from whole urine are likely to be quite different from those suitable for the relatively urea-free and unbuffered extracts used in these experiments. Furthermore, owing to the destruction by acid, much smaller yields of the pure compound are obtained by ethyl alcohol crystallization of the crude pregnandiol released by hydrolysis than can be obtained from the same amount of crude pregnandiol directly extracted without hydrolysis. It is anticipated that it will be possible to devise methods of hydrolysis which will be suitable for whole urine and which will not cause extensive destruction of the liberated pregnandiol. Owing to the impossibility of continuing this work at the present time, it was considered to be advisable to publish these preliminary results.

SUMMARY.

The presence of an ether-insoluble "combined" form of pregnandiol in the alkali-washed butyl alcoholic extract of human pregnancy urine has been detected. This complex is stable to hot alkali but is hydrolysed by hot acid. The optimum conditions for its hydrolysis by acids have been approximately determined.

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Note added 15 August, 1936. Since the completion of this paper Venning & Browne (*Proc. Soc. Exp. Biol. N.Y.* (1936), **34**, 792) have reported the isolation from human pregnancy urine of an oestriol monoglucuronide.