VI. A METHOD FOR THE ISOLATION OF PREGNANDIOL FROM HUMAN PREGNANCY URINE

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PREGNANDIOL, first isolated from human pregnancy urine by Marrian [1929], has now become a material of importance. Not only is it a starting material for the preparation of progesterone [Butenandt & Schmidt, 1934], but, since it is probably the excreted form of the hormone, its presence in the urine may be an index of progesterone production in the body. It is to these new interests that we owe the recent work of Venning & Browne [1936] and of Odell & Marrian [1936] who have investigated the separation of pregnandiol from butyl alcoholic extracts of urine. Venning & Browne isolated pregnandiolmonoglucuronide, also detected by Odell & Marrian, who investigated the conditions of hydrolysis of this compound. Butyl alcohol extraction is, however, difficult for large-scale operations and, failing access to suitable residues from the manufacture of oestriol from human pregnancy urine [cf. Remesov, 1935], an investigation has been made of the possibility of extracting pregnandiol from the by-products of the preparation of gonadotropic hormone. In the carrying out of this work British Drug Houses, Ltd. have given generous help by supplying material.

It has been found that in the method of preparing gonadotropic hormone from urine by adsorption on benzoic acid [Katzman & Doisy, 1932], all the free and the greater part of the combined pregnandiol are adsorbed at the same time. In this present work the conditions of precipitation of the benzoic acid and the conditions of hydrolysis and extraction of the adsorbed material have been studied with a view to developing a method of recovery of pregnandiol on a large scale.

EXPERIMENTAL

Benzoic acid precipitation of the urine

This part of the work, consisting essentially of the process of Katzman & Doisy [1932] for the isolation of gonadotropic material from urine of pregnancy, was carried out by British Drug Houses, Ltd. 1 kg. of sodium benzoate was added to each 45 l. of urine and, after adjusting the *pH* to approximately 3.6 by addition of HCl, the precipitated benzoic acid was collected. This precipitate was then stirred into 5 l. of acetone, the gonadotropic material was removed and the benzoic acid and other acetone-soluble material was recovered by evaporation of the filtrate. This benzoic acid residue and the benzoic acid-treated urine filtrate were placed at our disposal.

A. Benzoic acid residues

Extraction of free pregnandiol. The free pregnandiol was extracted directly from an alkaline solution of the crude benzoic acid. Toluene was chosen as the most suitable extractant on the grounds of its insolubility in water and comparative non-inflammability.

The crude benzoic acid was dissolved in a slight excess of NaOH (4 l. of 1% per kg. benzoic acid) and then extracted in a 12 l. continuous liquid-liquid extractor in which the aqueous phase was kept at 65–70° and was continuously stirred. Extraction of 300 mg. of pregnandiol from such a solution was complete, under these conditions, in 4 hours. All subsequent work was carried out in this apparatus using a standard extraction time of 8 hours.

The toluene extract was diluted to 3 l. and, after washing once with 500 ml. of 2N NaOH and four times with 400 ml. of water, it was evaporated under reduced pressure. The semi-crystalline residue was refluxed 5–10 min. with light petroleum and the mixture was placed in the ice-box for 4–5 hours. Recrystallization of the petroleum-insoluble material from acetone yielded pregnandiol melting at 231–234° which on recrystallization from ethyl alcohol melted at 237–237.5°. Using this technique, it was possible to obtain 90–150 mg. pregnandiol per kg. benzoic acid, equivalent to 2–3.3 mg. per l. of original urine.

Isolation of pregnandiol present in combination. Isolation of the pregnandiol present in combined form in the benzoic acid residues involved hydrolysis in order that it might be converted into the free, toluene-soluble form. On the basis of the work of Cohen & Marrian [1935] for the hydrolysis of combined oestriol in pregnancy urine, it appeared probable that autoclaving or boiling the material at pH 1.0 for 2 hours might prove satisfactory. Preliminary experiments soon showed that further study was necessary to determine the optimum conditions for hydrolysis.

The free pregnandiol was removed from 2.7 kg. of crude benzoic acid residues and the residual material, recovered on acidification of the extracted sodium benzoate solution, was divided into three equal parts. Each was suspended in 9 l. of HCl of suitable strength and hydrolysis was carried out. In each case, after removal of the liberated pregnandiol, the residue was rehydrolysed. The conditions of hydrolysis and yields of pregnandiol (m.p. 231–234°) obtained are given in Table I.

Table I. *Yields of pregnandiol on hydrolysis at 100 and 120°*

Batch	Strength of acid N	Treatment	Time hours	Yield mg.	Total yield mg.
I	0.12	Autoclaved 120°	2	100	—
Residue I	0.12	Re-autoclaved 120°	2	21	121
II	0.25	Autoclaved 120°	2	150	—
Residue II	0.25	Boiled	2	74	224
III	0.25	Boiled	4	226	—
Residue III	0.25	Autoclaved 120°	2	7	233

These results indicated that boiling rather than autoclaving the acidified material gave the better yields, and that a higher concentration of acid was required than was previously supposed. Hydrolysis of the crude benzoic acid residues was then studied, since a great deal of labour would be saved if this material could be processed directly without preliminary removal of the free pregnandiol to prevent its destruction.

A uniform mixture of 7 kg. of crude benzoic acid residues was divided into seven portions. Five of these were severally suspended in 10 l. of HCl of suitable strength, and hydrolysis was carried out. In the case of the sixth portion, the free pregnandiol was first removed and the residue was treated similarly to the other samples. The conditions of hydrolysis and yields of pregnandiol (m.p. 231–234°) of each batch are given in Table II.

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for (4l. of 1%...) *read* (4l. of 10%...)

Table II. *Yields of pregnandiol under varying hydrolytic conditions*

Batch	Strength of acid <i>N</i>	Treatment	Time hours	Yield mg.
I	0.30	Autoclaved 120°	2	100
II	0.12	Boiled	2	125
III	0.12	Boiled	4	186
IV	0.25	Boiled	2	241
V	0.25	Boiled	4	236
VI*	0.25	Boiled	2	126 (233)

* Free pregnandiol previously removed = 107 mg.

As a control the pregnandiol sodium glucuronate was isolated from 100 g. of the remaining sample by the method of Venning & Browne [1936]. Since full experimental details are lacking in their paper the modified procedure adopted is described in full, as follows.

The crude benzoic acid was taken up in excess of 2*N* NaOH and the solution was made just acid to litmus by addition of HCl. After dilution to 2 l., it was extracted three times with 400 ml. portions of butyl alcohol, these extracts being combined and washed twice with 200 ml. of *N*/3 NaOH and twice with 200 ml. portions of water. The butyl alcohol was removed under reduced pressure, the residue was taken up in 5 ml. of water, and 200 ml. of dry acetone were added. After standing overnight, the acetone-insoluble material was centrifuged off and, on dissolving in alcohol and taking to dryness under nitrogen, it gave a white, crystalline residue weighing 34.6 mg. and melting with decomposition at 265–266°. Recrystallization from ethyl alcohol gave the pure glucuronate which melted with decomposition at 270–272°. This quantity represented a pregnandiol content of 15–17 mg. or 150–170 mg. of combined pregnandiol per kg. of benzoic acid.

From these experiments it is seen that boiling the crude benzoic acid residues with 0.25 *N* HCl for 2 hours yields practically all the possible pregnandiol. It is evident that under these conditions the destruction of free pregnandiol is slight, since an increase in the time of boiling to 4 hours resulted in practically no decrease in the yield. These conditions of hydrolysis are more drastic than those recommended by Odell & Marrian for partially purified butyl alcoholic extracts. Using this technique, it was possible to obtain regularly yields of 235–440 mg. pregnandiol (m.p. 231–234°) per kg. of benzoic acid, equivalent to 5–10 mg. per l. of original urine. Recrystallization from ethyl alcohol gave pure pregnandiol melting at 237–237.5°, $[\alpha]_{5461}^{20} + 30.4^\circ$, $[\alpha]_D^{20} + 27.4^\circ$ in ethyl alcohol ($l=4$, $c=0.493\%$).

Adsorption of combined pregnandiol by benzoic acid. The effects of varying conditions on the adsorption of combined pregnandiol by benzoic acid were studied, and comparison of the yields of pregnandiolglucuronide was made with that obtained by the method of Venning & Browne. Seven 1 l. lots were taken from a composite sample of pregnancy urine (6th–8th months) and, after the addition of 25 g. of sodium benzoate to each, HCl was added to give the following values of pH: (a) 4.6; (b) 4.0; (c) 3.5; (d) 2.5; (e) 2.0; (f) 1.5; (g) 1.0. After standing for 2 hours, with occasional shaking, the precipitated benzoic acid was filtered off and the pregnandiolglucuronide isolated as its sodium salt by the method already described. The following yields of acetone-insoluble material were obtained: (a) 22 mg.; (b) 21 mg.; (c) 35 mg.; (d) 38 mg.; (e) 41 mg.; (f) 35 mg.; (g) 82 mg. All were crystalline white solids except (g) which was amorphous. (a) and (b) melted (with decomposition) at 256–258°, (c), (d), (e) and

(f) at 266–268°, while (g) had not melted at 295°. While recrystallization of (f) from 10 ml. of ethyl alcohol gave 12 mg. of pure glucuronide (m.p. 270–271°) similar treatment of (g) gave a very small amorphous precipitate. From this it was concluded that (g) contained little, if any, pregnandiolglucuronide. The results are shown graphically in Fig. 1.

Fig. 1. Adsorption of pregnandiolglucuronide by benzoic acid.

From this graph it is seen that maximum adsorption of the glucuronide occurs between pH 2.5 and 2.0.

As a control 1 l. of the same batch of urine was extracted directly with butyl alcohol and the acetone-insoluble material, on taking to dryness from alcoholic solution, gave a yellow glass weighing 112 mg. Recrystallization from ethyl alcohol gave 38 mg. of the crude glucuronate melting with decomposition at 264° (sintered 260°).

B. Urinary filtrate

Isolation of pregnandiol. Attention was next turned to the urine filtrates from the initial benzoic acid treatment. In many cases no free pregnandiol could be isolated by toluene extraction of this material after it had been made alkaline with NaOH, the highest yield of free pregnandiol obtained being 0.8 mg. per l. of urine. On the other hand 1–2 mg. pregnandiol per litre were present in the combined form.

Attempts to liberate the combined pregnandiol from these urine residues by direct hydrolysis gave unfavourable results. It was necessary to add a considerable excess of HCl above that amount required to adjust the pH to 1.0; otherwise a rise in pH occurred on boiling, due to hydrolysis of the urea present. In all such experiments the final product, recrystallized from acetone, melted at 215–218°, and repeated crystallization from this solvent or from ethyl alcohol failed to raise its melting-point, although a mixture with pure pregnandiol melted from 215 to 230°. Subsequent work showed it to be a mixture of pregnandiol and *allopreg*ndiol [Hartmann & Locher, 1935].

Repetition of the benzoic acid adsorption removed the remainder of the combined pregnandiol from the urine filtrate and on hydrolysis 1–2 mg. more pregnandiol per litre of urine were obtained. Attempts to concentrate the urine in an open steam pan led to destruction of the pregnandiol. This is illustrated by the following experiment.

200 l. of residual urine, after the first benzoic acid adsorption, were divided into two 100 l. batches, one being processed directly, the other being con-

centrated in an open steam pan to 25 l. 1 kg. of sodium benzoate was added to each of two 40 l. batches of the unconcentrated urine, the mixture was acidified to pH 2.0 and the benzoic acid was filtered off. After hydrolysis by boiling for 2 hours with 10 l. of 0.25 *N* HCl the freed pregnandiol was isolated in the manner already described except that the washed toluene residue was recrystallized directly from acetone since it contained no petroleum-soluble fraction. Yields of 67 and 62 mg. pregnandiol were obtained. Repetition of this procedure using two 10 l. lots of the concentrated urine failed to yield any pregnandiol.

Isolation of allopregnanediol. 900 mg. of low-melting material (m.p. 215–218°) from the acid hydrolysis of the urinary residues were acetylated and the product, on crystallization from ethyl alcohol, yielded 560 mg. crude pregnandiol diacetate (m.p. 172–174°) which on recrystallization from alcohol melted at 179–180°, $[\alpha]_{5461}^{20} + 42.5^\circ$, $[\alpha]_D^{20} + 34.3^\circ$ in benzene ($l=4$, $c=0.514\%$). Attempts to obtain further crystalline material from the mother-liquors using methyl alcohol or hexane as the solvent were unsuccessful. The residual acetates were therefore saponified by refluxing for half an hour with 5% methyl alcoholic KOH, diluted with water and ether-extracted. An ether-insoluble fraction was obtained which on recrystallization from aqueous alcohol yielded 121 mg. *allopregnanediol* melting at 241–243°. After recrystallization from methyl alcohol this had m.p. 243–245°, $[\alpha]_{5461}^{20} + 20^\circ$, $[\alpha]_D^{20} + 17^\circ$ in ethyl alcohol ($l=4$, $c=0.148\%$); the diacetate had m.p. 139.5–140.5°, $[\alpha]_{5461}^{20} + 21^\circ$; $[\alpha]_D^{20} + 18^\circ$ in benzene ($l=4$, $c=0.408\%$). On taking the ether extract to dryness a white solid melting at 190–200° was obtained which on recrystallization from acetone gave crystals melting at 212–220°.

DISCUSSION

The method described for the isolation of pregnandiol from pregnancy urine consists essentially of the concentration of the free and combined diol by adsorption on benzoic acid at pH 2.5–2.0. After acid hydrolysis of the combined material the pregnandiol is extracted from an alkaline solution of the adsorbate with toluene and separated from the other toluene-soluble material by washing with light petroleum and crystallization from acetone.

This process is particularly well suited for the handling of urine containing pregnandiol in small amount only, which is commonly the case with bulk collection. Concentration of the pregnandiol by benzoic acid adsorption is much less laborious and much cheaper, on a large scale, than extraction of the urine with a solvent such as butyl alcohol. While initial concentration of the urine would help to overcome this objection, such treatment would destroy the gonadotropic material, the other valuable product of the benzoic acid process. Further, if concentration is carried out at 100° destruction of the pregnandiol also takes place. Whilst extraction of the hydrolysed material is necessary, it is carried out on a greatly reduced volume of material and employs a much more economical solvent than butyl alcohol.

Attempts to isolate the pregnandiolglucuronide from the benzoic acid adsorbate proved unsuccessful unless the butyl alcohol extraction method of Venning & Browne was used. The acetone-insoluble material obtained by such treatment gave, on evaporation from an alcoholic solution, a crystalline residue of the crude sodium salt which melted at 266–268°. On the other hand the acetone-insoluble material, obtained by direct butyl alcohol extraction of the urine, on evaporation from alcoholic solution invariably yielded an amorphous residue or glass which required recrystallization from alcohol in order to obtain

the crystalline glucuronate. The final yields of crystalline product were about the same in each case. These findings indicate that preliminary benzoic acid treatment of the urine may prove of use for the quantitative measurement of the pregnandiolglucuronide present and this matter is being investigated further.

SUMMARY

The greater part of the pregnandiol, both free and combined, is removed from human pregnancy urine by adsorption on benzoic acid, the optimum reaction being between pH 2.5 and 2.0.

The combined pregnandiol thus adsorbed is best hydrolysed by boiling with 0.25 *N* HCl for 2 hours.

Pregnandiol appears to be destroyed by evaporation of the urine at 100° in an open vessel.

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