The Separation of Non-ketonic and Ketonic Sodium Glucuronidates from the 'Sodium Pregnanediol Glucuronide' of Human Pregnancy Urine: the Isolation of Pure Sodium Pregnane-3(α), 20(α)-diol Glucuronide*

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It has recently been shown (Marrion & Gough, 1946 *a, b*) that the 'sodium pregnanediol glucuronide' ('NaPG') prepared from human pregnancy urine by the method of Venning & Browne (1936) contains about 20 % of a water-soluble derivative of pregnan-3(α)-ol-20-one which cannot be removed by repeated recrystallization from 90 % (v/v) aqueous ethanol or by repeated precipitation from 50 % (v/v) aqueous acetone with 10 volumes of dry acetone. The assumption was made that this pregnanone derivative is probably the sodium salt of the glucuronide.

Since pure sodium pregnane-3(α), 20(α)-diol glucuronide was required in connexion with other work in progress in this laboratory, it was decided to attempt the separation of this substance from the 'NaPG' mixture and at the same time to attempt the isolation of the hitherto undescribed sodium pregnan-3(α)-ol-20-one glucuronide, the presence of which had been assumed.

RESULTS

At the outset various possible methods of separating the ketonic from the non-ketonic glucuronidates were considered and tested experimentally, using a qualitative Zimmermann test (procedure of Callow, Callow & Emmens, 1938) on 5 mg. quantities to detect the presence or absence of ketonic material in the fractions obtained.

Partition chromatography of the glucuronidic acids obtained by acidification of an aqueous solution of 'NaPG', on a potato starch column in moist butanolic solution (cf. Elsdon & Syngé, 1944) resulted in no separation of the ketonic and non-ketonic components.

By repeated recrystallization from ethanol of the 'triacetate lactone' obtained from 'NaPG' by acetylation at room temperature (Heard, Hoffman & Mack, 1944) a fraction was obtained which gave a less intense Zimmermann reaction than did the

original triacetate lactone, but the losses of material at each crystallization were so great and the degree of purification effected so small that it was evident that the method would be of little value for the preparation of pure sodium pregnanediol glucuronide in large quantities.

Separation of ketonic and non-ketonic glucuronidic acids by means of Girard's reagent T

Since it is possible to extract with ether the glucuronidic acids from an acidified solution of their sodium salts, the possibility was investigated of separating the ketonic and non-ketonic glucuronidic acids by means of trimethylammoniumacetohydrazide chloride (Girard's reagent T) according to the method of Girard & Sandulesco (1936). This method for the separation of ketonic from non-ketonic substances involves forming the trimethylammonium-acetohydrazone of the ketonic component by boiling the material with the reagent in 10 % (w/w) ethanolic acetic acid, followed by ether extraction of the non-ketonic component after neutralization of nine-tenths of the acetic acid and dilution with water. In preliminary trials of the method, in which the reagent T was omitted, it was found that the glucuronide linkage in 'NaPG' is stable under the conditions used for forming the hydrazone. However, it was not found to be possible to extract with ether the pregnanediol glucuronidic acid after neutralization of nine-tenths of the acetic acid, and it was therefore concluded that at the pH attained the pregnanediol glucuronidic acid is largely ionized. Accordingly the possibility of effecting the separation, omitting the partial neutralization of the acetic acid, was investigated.

Working under ice-cold conditions and as rapidly as possible in order to minimize the hydrolysis of the trimethylammoniumacetohydrazone, a 'non-ketonic' fraction was obtained which yielded, after two further successive separations in the same manner, a final product which gave a virtually negative Zimmermann reaction. After purification and drying at 137° a sodium salt, analyzing in good

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agreement with the formula $C_{27}H_{43}O_8Na$, was obtained (m.p. 283.5–284.5° corr., decomposition and evolution of gas). This gave a strongly positive Tollens reaction for glucuronic acid. The purified product was obtained in a yield of 28 % of the original 'NaPG' or 35 % of theory on the assumption that the 'NaPG' had contained 80 % of non-ketonic glucuronide. The low yield can probably be ascribed to incomplete extraction of the glucuronic acid from aqueous solution with ether.

The 'ketonic' fractions from each of the three successive Girard separations were recovered as their sodium salts, combined and subjected to two further Girard separations, the non-ketonic fractions obtained in each being discarded. The sodium salt of the final ketonic fraction yielded after purification a product which melted at 257–260° (corr., with decomposition and evolution of gas), gave an intense brick-red colour in the Zimmermann test, and a strongly positive Tollens reaction. Analysis suggested, however, that it was contaminated with inorganic material. The yield was 6 % of the original 'NaPG'.

The efficiency of the whole separation process and the purity of the products obtained was investigated by determining quantitatively the pregnanediol and 20-ketosteroid contents of the toluene-soluble material obtained from the original 'NaPG', and the non-ketonic and ketonic sodium glucuronides after acid hydrolysis. The methods used for these determinations were those described by Marrian & Gough (1946 b). It should be noted that these methods are not highly accurate. The results are shown in Table 1.

Table 1. *Analysis of glucuronide fractions*

Toluene-soluble hydrolysis product from	Pregnane-diol content (%)	20-Ketosteroid content (%)	M.p. (corr.) without purification
Original sodium pregnanediol glucuronide	77	23	211–220°
Non-ketonic sodium glucuronide	98	5.2	233–237°
Ketonic sodium glucuronide	23	85	127–147°

The pregnanediol and 20-ketosteroid contents of the unpurified hydrolysis product obtained from the non-ketonic sodium glucuronide indicate that this material is nearly pure pregnane-3(α), 20(α)-diol, a conclusion borne out by the melting-point, which was raised after recrystallization from ethanol to 236–238° (not depressed by admixture with an authentic sample of pregnane-3(α), 20(α)-diol).

These results, considered together with the results of the elementary analyses referred to previously, clearly show that the non-ketonic sodium glucuroni-

date obtained is nearly pure sodium pregnane-3(α), 20(α)-diol glucuronide. There are grounds for believing that this substance may not have been previously isolated in a state approximating to purity from human pregnancy urine.

In confirmation of the results obtained by Marrian & Gough (1946 b) with impure 'NaPG' it has been found that sodium pregnanediol glucuronide takes up 3H₂O in moist air, and that this trihydrate is converted to the anhydrous substance at 137° *in vacuo* over P₂O₅, whilst *in vacuo* over CaCl₂ at room temperature it loses 1.5H₂O.

The hydrolysis product obtained from the ketonic sodium glucuronide is not pure pregnan-3(α)-ol-20-one, since the melting-point before purification is low and since it gives a colour with concentrated H₂SO₄ corresponding to a pregnanediol content of about 20 %. This contaminant may be pregnanediol itself or some ketonic substance, which, unlike pregnan-3(α)-ol-20-one, is strongly chromogenic in the H₂SO₄ reaction but does not give a Zimmermann reaction. The ketonic sodium glucuronide obtained is clearly not a pure substance, although it is probably largely composed of sodium pregnan-3(α)-ol-20-one glucuronide, since the melting-point of the hydrolysis product was raised by purification to 145–148.5° (corr.) (not depressed by admixture with authentic pregnan-3(α)-ol-20-one). Attempts at the further purification of this ketonic sodium glucuronide fraction are now in progress.

Pregnane-3(α), 20(α)-diol glucuronic acid was obtained from the purified sodium salt and, after purification, melted at 178–180° (corr., with decomposition and evolution of gas), and had $[\alpha]_D^{20-25} - 5.0 \pm 0.5^\circ$. The acid which had been stored in a moist atmosphere lost 1.5H₂O *in vacuo* over CaCl₂ at room temperature, and analytical figures were then obtained in good agreement with those required for the anhydrous glucuronic acid, C₂₇H₄₄O₈.

EXPERIMENTAL

Separation and purification of the non-ketonic sodium glucuronide from 'NaPG'

'NaPG' (1.050g.) (m.p. 280°; corr., with decomposition and evolution of gas) was dissolved in 50 ml. of 10 % (w/w) ethanolic acetic acid and heated under reflux in a boiling water-bath for 1 hr. with 3 g. trimethylammoniumacetohydrazide chloride. After cooling under the tap and then in a freezing mixture, the solution was transferred to a chilled separating funnel with 500 ml. of chilled water and 250 ml. of chilled ether. After shaking, the aqueous layer was run off and re-extracted seven times as rapidly as possible with 250 ml. portions of chilled ether. The ethereal extracts were combined and extracted once with 100 ml. of 0.5N-NaOH and three times with 300 ml. portions of 0.1N-NaOH. The combined NaOH extracts were then extracted six times with a total volume of 2 l. butanol and the combined butanolic extracts allowed to stand in a

separating funnel until completely clear. After running off the small aqueous layer that separated, the butanolic extract was saturated with CO_2 and evaporated to dryness under reduced pressure.

The material so obtained was subjected to two further 'Girard' separations in succession in a similar manner. In these separations, however, the amount of trimethylammoniumacetohydrazide chloride was reduced to 2.5 g. and the volume of ethanolic acetic acid to 30 ml. The volumes of water, ether and NaOH solution, but not of butanol, were reduced proportionately. The final butanolic extract obtained after the third separation was washed twice with 1/12 volume of water before evaporating to dryness. A yield of 0.436 g. of crude non-ketonic sodium glucuronide was obtained. This was dissolved in 20 ml. of 50% (v/v) aqueous acetone and precipitated by the addition of 200 ml. of dry acetone. The precipitate was filtered with suction and washed with dry acetone. After a second precipitation by 10 volumes of dry acetone from solution in 16.5 ml. of 50% acetone a product weighing 0.305 g. was obtained.

This material was combined with 0.344 g. of a second batch of purified non-ketonic sodium glucuronide obtained from 0.986 g. of 'NaPG' by the same procedure. The combined batches were dissolved in 34 ml. of 50% acetone and precipitated with 340 ml. of dry acetone. The precipitate was collected in the usual way and dried at room temperature *in vacuo* over CaCl_2 . A total yield of 0.572 g. from 2.036 g. 'NaPG' was obtained. This purified non-ketonic sodium glucuronide melted at $283.5\text{--}284.5^\circ$ (corr., with decomposition and evolution of gas) and gave a virtually negative Zimmermann reaction on 5 mg. It gave a strongly positive Tollens reaction for glucuronic acid.

Weight after exposure to moisture-saturated atmosphere for 15 hr., 0.1735 g. (a). Weight after drying to constant weight *in vacuo* over CaCl_2 at room temp., 0.1649 g. (b). Weight after drying for 10 hr. *in vacuo* over P_2O_5 at 137° , 0.1568 g. (c). Weight after re-exposure to moist atmosphere for 24 hr., 0.1741 g. (d). Weight after redrying to constant weight *in vacuo* over CaCl_2 at room temp., 0.1641 g. (e). Moisture content of CaCl_2 dried substance = $(b - c)/b \times 100 = 4.9$; $(e - c)/e \times 100 = 4.4\%$. Moisture content of fully hydrated substance = $(a - c)/a \times 100 = 9.6$; $(d - c)/d \times 100 = 9.9\%$. Calc. for $\text{C}_{27}\text{H}_{43}\text{O}_5\text{Na}$, 1.5 H_2O ; 4.9% H_2O ; $\text{C}_{27}\text{H}_{43}\text{O}_5\text{Na}$, 3 H_2O : 9.4% H_2O . 2.389 mg. dried at 137° for 10 hr. *in vacuo* over P_2O_5 gave 5.319 mg. CO_2 , 1.853 mg. H_2O , 0.253 mg. Na_2CO_3 and 0.02 mg. unburned C. Found: C, 62.7; H, 8.7; Na, 4.6. Calc. for $\text{C}_{27}\text{H}_{43}\text{O}_5\text{Na}$: C, 62.5; H, 8.4; Na, 4.4%.

Separation of the ketonic sodium glucuronide from 'NaPG'

The ketonic fractions from each of the three separations with trimethylammoniumacetohydrazide chloride described in the preceding section were obtained in the following manner: the aqueous phase after removal of the non-ketonic fractions by ether extraction was acidified with concentrated HCl (2.0 ml./100 ml. solution). After standing at room temperature for 1 hr., the mixture was made just neutral to litmus by the addition of 10N-NaOH, and then an excess of 1.0 ml. of 10N-NaOH was added/100 ml. This alkaline solution was extracted six times with a total volume of 1 l. butanol, and the extract allowed to stand in a

separating funnel until clear. The small aqueous layer that separated was run off and the butanol evaporated to dryness after saturating with CO_2 .

The three ketonic fractions so obtained were combined and subjected to two further successive separations with trimethylammoniumacetohydrazide chloride. The reactions were carried out as previously described in 30 ml. of 10% (w/w) ethanolic acetic acid with 2.5 g. of the reagent. The non-ketonic fractions obtained from these separations were rejected. The butanolic extract containing the ketonic fraction from the last separation was washed twice with 1/12 volume of water and evaporated to dryness under reduced pressure. The crude ketonic fraction thus obtained was dissolved in 15 ml. of 50% (v/v) aqueous acetone and precipitated with 150 ml. of dry acetone. After a second precipitation from 10 ml. of 50% acetone with 10 volumes of dry acetone a yield of 0.115 g. of ketonic sodium glucuronide was obtained.

This material was combined with 0.063 g. of a second batch of ketonic sodium glucuronide obtained from 0.986 g. of 'NaPG' by the same procedure. The combined batches were given a final purification by dissolving in 15 ml. of 50% acetone, filtering and then precipitating with 150 ml. of dry acetone. From 2.036 g. 'NaPG', 0.131 g. of ketonic sodium glucuronide melting at $257\text{--}260^\circ$ (corr., with decomposition and evolution of gas) was obtained. The product gave an intense brick-red colour in the Zimmermann reaction and a strongly positive Tollens test for glucuronic acid.

2.470 g. dried at 137° for 10 hr. *in vacuo* over P_2O_5 gave 5.201 mg. CO_2 , 1.782 mg. H_2O , and 0.333 mg. Na_2CO_3 . 1.994 mg. similarly dried gave 4.193 mg. CO_2 , 1.449 mg. H_2O , and 0.271 mg. Na_2CO_3 . Found: C, 58.9 and 58.8; H, 8.1 and 8.1; Na, 5.8 and 5.9. Calc. for $\text{C}_{27}\text{H}_{41}\text{O}_5\text{Na}$: C, 62.7; H, 8.0; Na, 4.5%.

Hydrolysis of 'NaPG' and of the separated non-ketonic and ketonic sodium glucuronides

Samples of approximately 100 mg. each of the original 'NaPG' of the non-ketonic sodium glucuronide and of the ketonic sodium glucuronide were each treated as follows. The sample was suspended in 250 ml. of water and 65 ml. of toluene added. The mixture was heated to boiling under reflux and then 25 ml. of conc. HCl added. Boiling was continued for 10 min. and the flask then cooled under the tap. The mixture was transferred to a separating funnel, shaken thoroughly and the aqueous layer run off. This was re-extracted four more times with 65 ml. portions of toluene. The combined toluene extracts were washed once with 50 ml. of 5% (w/v) NaHCO_3 , twice with 50 ml. portions of water, and evaporated to dryness.

Pregnanediol was determined in the hydrolysis products by the Talbot, Berman, MacLachlan & Wolfe (1941) procedure as described by Marrian & Gough (1946b). 20-Ketosteroids were determined as described by the latter authors.

'NaPG' (99.8 mg.) yielded 54.2 mg. of toluene-soluble hydrolysis product melting at $211\text{--}220^\circ$ (corr.). 0.254 mg. of this product gave a colour in the H_2SO_4 reaction equivalent to the colour given by 0.197 mg. of authentic pregnane-3(α), 20(α)-diol. 1.016 mg. gave a colour in the Zimmermann reaction equal to that given by 0.239 mg. of authentic pregnan-3(α)-ol-20-one.

The non-ketonic sodium glucuronide (103.2 mg.) yielded 62.0 mg. of toluene-soluble hydrolysis product melting at 233–237° (corr.). 0.243 mg. gave the same intensity of colour in the H_2SO_4 reaction as 0.239 mg. of authentic pregnanediol, while 1.943 mg. gave the same intensity of colour in the Zimmermann reaction as 0.102 mg. authentic pregnanolone. After one recrystallization from ethanol the melting-point was 236–238° (corr.) and this was not depressed by admixture with authentic pregnane-3(α), 20(α)-diol (m.p. 235–237°, corr.).

From 101.4 mg. of the ketonic sodium glucuronide there were obtained 62.0 mg. of toluene-soluble hydrolysis product melting at 127–147° (corr.). In the H_2SO_4 reaction 1.486 mg. gave a colour equivalent to that given by 0.342 mg. of pregnanediol, and in the Zimmermann reaction 0.239 mg. gave a colour equivalent to that given by 0.202 mg. of pregnanolone. The product was purified by adsorption from benzene solution on an Al_2O_3 (6×1.3 cm.) column and elution with ether. The product so obtained after two crystallizations from *n*-hexane melted at 145–149° (corr.). Mixed with authentic pregnan-3(α)-ol-20-one (m.p. 147–149.5°, corr.) the melting point was 146.5–149.5° (corr.).

Pregnane-3(α), 20(α)-diol glucuronidic acid from its sodium salt

The purified non-ketonic sodium glucuronide (0.173 g.) was dissolved in 60 ml. of moist butanol with warming. The solution was acidified with 2 ml. of glacial acetic acid, transferred to a separating funnel, washed three times with 5 ml. portions of water, and finally evaporated to dryness under reduced pressure. The residue was heated to boiling with 5 ml. of water and the mixture filtered hot. The insoluble material was leached twice more in the same way with 5 ml. portions of hot water and then dissolved in 20 ml. of hot 25% (v/v) aqueous ethanol. The hot solution was filtered, evaporated under reduced pressure to the point of incipient crystallization and then set aside in the refrigerator overnight. The well-formed but slightly buff-coloured crystals that separated were collected by centrifugation, washed once in the centrifuge tube with 10 ml. of cold water and dried at room temperature over P_2O_5 *in vacuo*. A yield of 129 mg. of material melting at 178–180° (corr., with decomposition and evolution of gas) was obtained.

Material weighed after exposure to a moist atmosphere (2.160 mg.) was dried to constant weight *in vacuo* over CaCl_2 at room temperature. Weight loss observed, 0.122 mg. or 5.6%. Similarly treated, 10.497 mg. lost 0.581 mg. or

5.5%. Calc. for $\text{C}_{27}\text{H}_{44}\text{O}_8$, $1.5\text{H}_2\text{O}$: 5.2% H_2O . The m.p. was unchanged after drying.

A solution of the anhydrous substance (9.916 mg.) in 0.50 ml. ethanol gave a rotation of -0.049° (standard error 0.0054°) in a 0.5 dm. tube. Hence $[\alpha]_D^{20.5} = -5.0 \pm 0.5^\circ$.

2.038 mg. dried at room temperature over CaCl_2 *in vacuo* for 70 hr. gave 4.866 mg. CO_2 and 1.655 mg. H_2O . 2.378 mg. similarly dried gave 5.688 mg. CO_2 and 1.923 mg. H_2O . Found: C, 65.1 and 65.2; H, 9.1 and 9.1. Calc. for $\text{C}_{27}\text{H}_{44}\text{O}_8$: C, 65.3; H, 8.9%.

SUMMARY

1. 'Sodium pregnanediol glucuronide' prepared from human pregnancy urine has been separated into non-ketonic and ketonic sodium glucuronide fractions by means of trimethylammoniumacetohydrazide chloride.

2. The non-ketonic sodium glucuronide appears to be nearly pure sodium pregnane-3(α), 20(α)-diol glucuronide, and is contaminated with traces only of ketonic material.

3. Sodium pregnane-3(α), 20(α)-diol glucuronide dried under different conditions is obtained anhydrous or with 1.5 or 3 molecules of water of crystallization.

4. Pregnane-3(α), 20(α)-diol glucuronidic acid has been obtained from its sodium salt. Under different conditions of drying it is obtained anhydrous or with 1.5 molecules of water of crystallization.

5. The ketonic sodium glucuronide obtained appears to consist mainly of the hitherto undescribed sodium pregnan-3(α)-ol-20-one glucuronide; it has not, however, so far been obtained in a pure state.

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