The Nature of Pregnanediol-like Glucuronide

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A method was recently described (Bisset, Brooksbank & Haslewood, 1948) for the estimation of a fraction of urinary glucuronide with solubilities resembling those of pregnanediolglucuronidic acid and its salts. Analyses of normal men's urine and urine of women in the 'follicular' phase of the menstrual cycle gave values (expressed as pure sodium pregnanediolglucuronide, NaPG) of glucuronide output higher than those obtained for NaPG by other workers. The total glucuronide fraction (entrained on barium phosphate) was designated 'pregnanediol-like glucuronide (PLG)', and we decided to extract the material in larger quantities and to attempt to determine its precise chemical nature.

Table 1 lists steroids which have already been obtained from glucuronide mixtures or from single

We now describe the isolation from urine of a glucuronide fraction resembling as closely as possible the PLG estimated by the procedure of Bisset *et al.* (1948), and the investigation of material resulting from its hydrolysis. Part of the work has already been reported in a preliminary communication (Brooksbank & Haslewood, 1949).

We decided to use bacteria for the hydrolysis of the glucuronide mixture after preliminary experiments had suggested that acid hydrolysis led to extensive destruction. Concurrent research on the occurrence of glucuronide-splitting ability in micro-organisms had demonstrated that certain strains of *Staphylococcus albus* are particularly active in this respect (Barber, Brooksbank & Haslewood, 1948), and one of these active strains was employed for the large-scale hydrolysis of PLG.

Table 1. *Steroids isolated from urinary glucuronides*

Steroid	Urine	Authors
Oestriol	Human pregnancy	Cohen & Marrian, 1936
Pregnane-3 α :20 α -diol	Human pregnancy	Venning & Browne, 1936
Pregnane-3 α :20 α -diol	Human pregnancy	Sutherland & Marrian, 1947
Pregnane-3 α :20 α -diol	Human female non-pregnancy	Venning, Henry & Browne, 1937
Pregnane-3 α :20 α -diol	Human male	Westphal, 1944
Pregnane-3 α -ol-20-one	Human pregnancy	Marrian & Gough, 1946
Pregnane-3 α :17 α -diol-20-one	Human female pseudo-hermaphrodite	Mason & Strickler, 1947
Pregnane-3 α :17 α :20 α -triol	Human female (adrenal cortical hyperplasia)	Mason & Kepler, 1945

glucuronides extracted from the urines named. The presence of conjugated 17-ketosteroids in urinary glucuronide fractions was suggested by the results of Allen & Viergiver (1941). In addition, Mason (1948) has described experiments which indicate the existence in the urine examined of several more steroids as glucuronide conjugates. He subjected *n*-butanol extracts of urine (that of a woman with an adrenal cortical tumour), freed of free steroids, to enzymic hydrolysis, using rat-liver powder known to contain β -glucuronidase and no sulphatase. The following free steroids were liberated partially or completely in this way: androsterone, aetiocholan-3 α -ol-17-one, aetiocholan-3 α :17 β -diol (referred to by Mason (1948), as a 3:17 α -diol), pregnane-3 α :20 α -diol, a compound T, C₁₉H₃₃O₂, m.p. 207–209°, and a compound C₂₁H₃₄O, m.p. 145°. These experiments do not, of course, exclude the possibility that the compounds mentioned were liberated from some unknown type of conjugate.

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METHODS

Melting points are uncorrected. The micro-analysis was done by Drs Weiler and Strauss, Oxford.

Abbreviations. NaPG(V) = a crystalline specimen of glucuronide, prepared by the method of Venning (1937, 1938); NaPG(M) = purified glucuronide, freed from ketonic material (Sutherland & Marrian, 1947); NaPG = anhydrous Na pregnanediolglucuronide; PLG = pregnanediol-like glucuronide, i.e. strictly, material estimated by the method of Bisset *et al.* 1948; the term is, however, used to denote the material extracted and investigated on the large scale.

Light petroleum used was of b.p. 40–50°. Al₂O₃ for adsorption was obtained from Hopkin and Williams Ltd.

Estimation of glucuronide in crude PLG, etc.

The estimation of glucuronide entrained on Ba phosphate precipitates, of glucuronide in crude batches of PLG recovered from these precipitates and from chromatographic columns was performed by heating directly with naphthoresorcinol and HCl as described by Bisset *et al.* (1948). Thus, for the estimation of glucuronide entrained on Ba phosphate, samples (in duplicate) expected to contain not more than 0.2 mg. of glucuronide (as NaPG) were weighed

out in test tubes (15 × 2 cm.) and dissolved in 2 ml. of 2N-HCl. To each tube were then added water (1.4 ml.), 0.05M-K₃Fe(CN)₆ (0.1 ml.), 9N-HCl (1.5 ml.) and 2 ml. of aqueous 0.025M-naphthoresorcinol solution (freshly made up and filtered). Colour development and measurement were then carried out as previously described. For the estimation of glucuronide in crude *PLG* samples, 2–4 mg. of the sample were weighed (in duplicate) and dissolved in water (20 ml.). This usually gave a solution of which 1.0–1.4 ml. contained the amount of glucuronide (0.05–0.2 mg.) necessary for accurate measurement by our modification of the naphthoresorcinol reaction, using the quantities of reagents given above.

Assay figures quoted throughout are in terms of pure anhydrous NaPG. However, *PLG* samples were always hygroscopic, apparently becoming more so as they were purified, and it was therefore not possible to weigh the samples accurately. Errors due to uptake of water were minimized by allowing the samples to stand 10–15 min. between removal from the vacuum desiccator in which they were kept and weighing. Duplicate assays then rarely differed by more than 10%. Nevertheless, the hygroscopic nature of *PLG* samples made it impossible to determine their true glucuronide content precisely. Even allowing for the fact that the molecular weights of a considerable proportion of the *PLG* constituents (e.g. androsterylglucuronide) may be lower than that of NaPG, the actual figures of percentage purity quoted probably err on the low side on account of the uptake of water.

In the colorimetric estimations, NaPG(V) solution was sometimes used as standard; this was in turn standardized against NaPG(M). More usually the grey solution described by Thomson (1946) was used as artificial standard, as it was more stable than NaPG(V) solutions.

Preparation of *PLG*

Collection and extraction of urine. Fresh unpreserved pooled men's urine was collected in lots of 4–8 l. Each lot was shaken with Zn acetate (96 g.), filtered, and 4 l. of the filtrate extracted with *n*-butanol (2 l., purified and tested for glucuronide-destroying impurities as previously described by Bisset *et al.* 1948). In order to save time and solvent, only one extraction with butanol was performed on each batch of urine, and the same butanol (with the volume adjusted with fresh butanol to 2 l. after each use) was employed for a total of five extractions. The butanol extract, which then represented 20 l. of urine, was washed with 0.2N-NaOH (900 ml.) and then with 2% (w/v) NaCl solution (2 × 180 ml.). The butanol was then evaporated to dryness *in vacuo*, ethanol being used to remove the last traces.

The residue contained usually about 100 mg. of *PLG* at this stage. This was determined on a number of batches by dissolving the residue in water to give a glucuronide concentration judged to be of the order of 0.1–0.2 mg./ml. and performing quantitative entrainment and glucuronide estimations on suitable samples (Bisset *et al.* 1948).

Entrainment of PLG. The residue from butanol extraction of 20 l. of urine was dissolved in water to give a concentration of *PLG* which was near to that obtaining in the diluted filtrate (*F*) used for entrainment in the quantitative procedure (Bisset *et al.* 1948), but which would give a consistent and fair yield of *PLG* by entrainment. In preliminary work with residues from 20 l. of urine, 80 times the quantities of water and of precipitating reagents used in

the small-scale quantitative procedure (the corresponding residue of which is equivalent to 250 ml. of urine) gave unsatisfactorily low and inconsistent yields of *PLG*. We therefore used volumes of water and of precipitating reagents 50 times those used on the small scale. The butanol residue was dissolved in 1 l. of water and treated with 250 ml. each of BaCl₂ (0.5M) and Na₂HPO₄ (0.2M, containing 50 ml. N-NaOH/l.).

To ensure complete solution of the *PLG* the extract was warmed to 95° and cooled to room temperature before entrainment. If the thymol-salicylic acid mixture had been used as preservative, as was done with women's urine, much of it came through at this stage. The aqueous extract was in these cases washed with ethyl ether (separated by centrifugation) before entrainment of *PLG* was carried out (*PLG* was insoluble in ethyl ether).

After entrainment, the Ba phosphate precipitate was filtered off under suction and washed by stirring with acetone (300 ml., redistilled over CaO). The precipitate was then sucked dry and kept *in vacuo* over H₂SO₄.

Recovery of PLG from barium phosphate precipitates. Before the *PLG* was removed from the Ba phosphate it was further purified by extracting batches of 20–23 g. of Ba phosphate precipitate, each carrying about 200 mg. of *PLG*, with absolute ethanol for 2 days in a Soxhlet apparatus. Estimations on the residue from the ethanol used in these extractions never showed the presence of more than traces of glucuronide.

In the initial work the *PLG* was recovered from the Ba phosphate by exhaustive Soxhlet extraction with water, followed, after the addition of Na₂SO₄ solution to precipitate Ba⁺⁺ ions, by extraction of the water with butanol. However, at least 4 days' extraction of the precipitate was necessary, and losses of up to 20% of the *PLG* were experienced. For this reason, the much quicker procedure was applied of dissolving the Ba phosphate in acid (for 20–23 g. batches, 150 ml. of N-HCl, quickly diluted to 0.5N) and extracting the acid solution without delay with purified *n*-butanol (120, 60 and 60 ml.). The butanol was washed once with 25 ml. of 2% (w/v) NaCl solution and shaken vigorously with excess solid NaHCO₃. The mixture was left overnight, washed twice with 25 ml. of water, filtered and evaporated to dryness at 50–55°/20 mm. Hg. The dry amorphous residue was dark brown and contained a small amount of inorganic material. It was extracted with warm methanol (30–40 ml., redistilled over KOH) and the methanol solution transferred to a weighed test tube and evaporated at 50–55° under a stream of N₂. The residue was scratched with 1–2 ml. of ice-cold dry acetone, which was filtered off and discarded. The acetone removed a small quantity of impurities and, if absolutely dry, negligible amounts of glucuronide. The acetone-insoluble material was collected and kept *in vacuo* over H₂SO₄; it was a concentrated preparation of crude *PLG*. From men's urine, about 8 mg. of this produce per litre were obtained. It assayed at 45–55% of glucuronide (as NaPG). Thus about 4 mg. of pure *PLG* (as NaPG) were obtained per litre of urine.

PLG from women's urine. Three 20 l. batches of women's urine were collected, in Winchester bottles each containing a mixture of powdered thymol (4 g.) and salicylic acid (2 g.) as preservative, from the 4th to 14th day of the menstrual cycle and pooled. The urine was extracted in the same way as described for men's urine. The amount of *PLG* was only about half that extracted from men's urine under the same conditions, and entrainment on Ba phosphate was carried

out on the 2nd and 3rd batches at double the concentration of residue in order to obtain a high yield of *PLG*; the butanol residue was dissolved in 500 ml. of water in the 2nd batch, and in 375 ml. of water and 125 ml. of 2% (w/v) NaCl solution in the 3rd batch, with the subsequent addition of 125 ml. each of the BaCl_2 and Na_2HPO_4 solutions in each case. The *PLG* derived in this way from women's urine therefore probably differed from that furnished by men's urine in containing extra, possibly non-steroidal, glucuronide such as is estimated in the quantitative procedure by the use of 4 ml. of extract at entrainment (Bisset *et al.* 1948). The final crude *PLG* obtained from 61 l. of urine weighed 311 mg. and assayed at the high figure of 69% of glucuronide. It did not differ in obvious properties from *PLG* obtained from men's urine.

Attempts at further purification of crude PLG. No very thorough study of the possibilities of purifying *PLG* by solvent partition or precipitation methods was made, but in a few experiments it was found that the following manoeuvres gave no appreciable purification and at the same time resulted in some loss of the material: extraction with hot ethanol or methanol (this was partially effective in purifying samples containing much inorganic material), precipitation from water with acetone, precipitation from methanol with ether (cf. Jayle & Libert, 1946), precipitation from methanol with acetone and precipitation from water with ethanol. In one experiment, extraction with CHCl_3 did seem to bring about considerable purification, although the glucuronide was appreciably soluble in CHCl_3 .

We therefore attempted the purification of *PLG* by adsorption chromatography, hoping that as well as the removal of glucuronides from non-glucuronide material a partial separation of the glucuronide components of *PLG* might be effected. However, the glucuronide tended to be destroyed on the column (i.e. material giving a blue colour in the naphthoresorcinol reaction was 'lost' on the column). This destruction occurred more particularly when a high ratio of adsorbent to material adsorbed was used.

The progress of the column (no. 2, Table 5, see p. 41) which gave the purest *PLG* obtained is given in Table 2. In later columns more methanol and less 1:1 (v/v) butanol/methanol were used for elution, as the aim was to obtain

large yields of reasonably pure glucuronide for hydrolysis experiments. Ethanol was relatively ineffective as an eluting solvent.

Hydrolysis of *PLG*

Acid hydrolysis. This was attempted with dilute HCl at room temperature and also with heating. No crystalline substances could be easily isolated, and the ether-soluble products appeared to have been partially decomposed.

Bacterial hydrolysis. A strain of *Staph. albus* known to have glucuronide-destroying activity was selected for large-scale work. A preliminary experiment was performed on NaPG. NaPG(V) (100 mg., equivalent to 97.1 mg. of NaPG) was dissolved in water (40 ml.) and the solution added to Lablemco peptone broth (200 ml.). The mixture was sterilized by autoclaving, seeded with the organism, and incubated for 1 week at 37°. After autoclaving, the mixture was filtered, and the filter paper carrying the precipitated material was extracted for 2 days with ethanol in a Soxhlet apparatus. The total ethanol-soluble material weighed 50.9 mg. From this material crystals were obtained (from the ethanol) which melted at 225–229°; mixed melting point with authentic pregnane-3 α :20 α -diol, 226–229°. No attempt was made to isolate all the pregnanediol (nor was any pregnane-3 α -ol-20-one isolated), but if all the ethanol-soluble material was pregnanediol, this would represent 85% of the steroid moiety of the added conjugate. The absence of any conjugated glucuronide after incubation was confirmed by carrying out the glucuronide entrainment and estimation procedures on the residue from extraction of the filtered incubation mixture with butanol.

We therefore employed the same strain of *Staph. albus* to hydrolyse two batches of *PLG*. In the first experiment 151 mg. of the best chromatographed *PLG*, assaying at 76% and therefore containing 115 mg. of glucuronide (calculated as NaPG), were dissolved in water (40 ml.) and Lablemco peptone broth (200 ml.). This solution was sterilized by heating, seeded with an active strain of *Staph. albus* and incubated at 37°. At the end of incubation for 1 week it was again sterilized by autoclaving. (All the above bacteriological work was kindly executed by Dr Mary Barber at St Thomas's Hospital Medical School, London.)

Table 2. Chromatogram of *PLG* from male urine

(103.3 mg. of solid, containing 52.6 mg. of *PLG* (as NaPG) were put on to a column of Al_2O_3 (1 g.; approx. 5 × 50 mm.) with 1:1 (v/v) butanol/methanol.)

Fraction	Eluted with		Eluate		<i>PLG</i> content (as NaPG) (%)
	Vol. (ml.)	Solvent	Wt. (mg.)	Appearance	
1	25	1:1 (v/v) butanol/methanol	35.8	Yellow, glassy	7.9
2	20	1:1 (v/v) butanol/methanol	6.7	Nearly white, solid	28.9
3	20	1:1 (v/v) butanol/methanol	6.6	Nearly white, solid	60.1
4	30	1:1 (v/v) butanol/methanol	4.2	Nearly white, solid	77.5
5	25	1:1 (v/v) butanol/methanol	5.0	Nearly white, solid	62.2
6	20	Methanol	13.3	Yellow, glassy (very hygroscopic)	92.7
7	100	Methanol	15.7	Brownish, amorphous	90.5
8	100	Methanol	3.1	Brownish, amorphous	92.2
9	100	Methanol	4.0	Brownish, slightly gummy	35.9
10	50	1% (v/v) acetic acid/methanol	7.6	Brown, glassy, gummy	30.2
Total solid recovered			102.0 mg. = 98.7%		
Total <i>PLG</i> recovered			48.3 mg. = 91.8%		

The autoclaved mixture, which contained a precipitate, was extracted with ether for 8 hr. in a continuous extractor. The ether extract gave a residue weighing approx. 30 mg., which appeared to contain some crystals. The aqueous layer was exhaustively extracted in a separating funnel with ethyl acetate. The ethyl acetate residue (approx. 40 mg.) was a gum, which showed a tendency to crystallize when treated with light petroleum. Both residues were combined (with ethanol) and together weighed 72.7 mg. All but an insoluble trace of this was dissolved in benzene and run on to Al_2O_3 (0.5 g.) in a column. Fraction 1 (eluted with 50 ml. benzene) weighed 37.4 mg., possessed a strong odour of musk and crystallized when treated with light petroleum. The second fraction (eluted with 50 ml. of 1:1 (v/v) ether/benzene) weighed 7 mg. and was crystalline and slightly odorous. Thereafter 1:1 (v/v) ether/benzene (50 ml.), ether (50 ml.) and ethanol (50 ml.) failed to elute any more material, so that about 28 mg. remained unaccounted for in this experiment. Fraction 1 was put on to Al_2O_3 (0.5 g.) in a column with, at first, approx. 50 ml. of light petroleum, in which it did not entirely dissolve. The bulk came through in the first fraction and was combined with a very small additional amount eluted with a further 50 ml. of light petroleum to give 22.7 mg. of very easily crystallizable material. This appeared to contain hydrocarbon-like substances, which were partly removed by the addition of a few drops of methanol to a solution in 1:1 (v/v) methanol/ethanol. The wax-like material separated out and was filtered off. The substance obtained by evaporation of mother liquor, which was the odorous, easily crystallizable material, was soluble in all the common organic solvents. It was finally crystallized from dilute acetone, and then from a very small quantity of light petroleum. Sublimation at $125^\circ/0.3$ mm. Hg gave material again recrystallized from light petroleum to yield long white needles, m.p. $143\text{--}143.5^\circ$, with a strong odour. (Found: C, 83.0; H, 11.3. Calc. for $\text{C}_{19}\text{H}_{30}\text{O}$: C, 83.2; H, 11.0%.) A sample of authentic androst-16-en-3 α -ol (kindly sent to us by Prof. V. Prelog of Zürich; cf. Prelog & Ruzicka, 1944) resembled our substance closely and did not depress its melting point.

For the second bacterial hydrolysis 335 mg. of *PLG* assaying at 70% (235 mg. of glucuronide, calculated as *NaPG*) was dissolved in water (40 ml.), made up to 400 ml. with Lablemco peptone broth, and the sterilized mixture incubated for 6 days with the same strain of *Staph. albus* as in the previous experiment and finally autoclaved. A strong smell of musk was noted in the crude mixture even at this stage. The mixture was filtered, and the filter paper was extracted in a Soxhlet apparatus for 2 days with ether. The filtrate was shaken with ether in a separating funnel. The residue from the combined ether extracts weighed 114.2 mg.

To test for unchanged glucuronide conjugate, 10 ml. of the final aqueous filtrate were made up to 25 ml. with water, and the solution extracted with 10, 5 and 5 ml. of butanol. The butanol was washed with 0.2N-NaOH (10 ml.) and 2% (w/v) aqueous NaCl (2+2 ml.) evaporated to dryness and the residue dissolved in water (10 ml.). Determination of glucuronide was performed on this aqueous extract both with and without entrainment. No glucuronide was entrained, showing that no unhydrolysed *PLG* remained in the incubated mixture. Estimation without entrainment showed an amount of 40 mg. of butanol-soluble glucuronide (as *NaPG*) in the total filtrate from the incubation mixture. Direct assay on the filtrate itself gave a figure of 122 mg., or

51.9% of the original amount of glucuronide (as *NaPG*); therefore, 32.8% of the total glucuronide present after bacterial hydrolysis was extractable with butanol.

The total ether-soluble material (114.2 mg.) extracted from the incubation mixture was dissolved in ethanol (2 ml., distilled over Na) and the solution refluxed for 1.5 hr. after the addition of A.R. acetic acid (0.2 ml.) and Girard's reagent T (0.1 g.). Some ethanol evaporated through a leak; further amounts of ethanol (2-3 ml.) and Girard's reagent T (0.1 g.) were added. The mixture was refluxed for a further 20 min., stoppered and left overnight. The non-ketonic fraction was extracted with ether after the addition of water to the mixture and neutralization to pH 7.6 (bromthymol blue); it weighed 76.0 mg. The ketonic fraction, extracted with ether from the aqueous solution after removal of non-ketonic material and acidification, weighed 11.0 mg. Therefore 27.2 mg. of the starting material were unaccounted for. The ketonic fraction was refluxed in ethanol (1 ml.) for 1 hr. with semicarbazide hydrochloride (20 mg.) and Na acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$; 30 mg.); no semicarbazone was precipitated.

The non-ketonic fraction (76 mg.) was warmed with 10 ml. of a solution of digitonin (1 g. in 90 ml. ethanol + 10 ml. water). The dried digitonide weighed 12.2 mg. It was decomposed with pyridine (1 ml.) and ether, and the ether-soluble material weighed 4.8 mg. This ' β -fraction' was semi-crystalline, but gave only a gum when sublimed in high vacuum. The digitonin non-precipitable fraction weighed 71.2 mg. Of this, 60 mg. were subjected to exhaustive fractionation on alumina. It was dissolved in benzene (5 ml.) and light petroleum was added until cloudiness just appeared; the solution was then poured on to a column of Al_2O_3 (3 g.). The solvent was then approx. 70% (v/v) benzene/light petroleum.

The progress of the column is shown in Table 3.

Fractions 1 and 2 were combined and separated again on a column, with 90% (v/v) light petroleum/benzene. The bulk came through in the first fraction as a gum, whilst 2.6 mg. of crystalline, musk-smelling material were eluted with 10 ml. 70% light petroleum/benzene.

Therefore, including fractions 3, 4 and 5, about 14 mg. of musk-smelling crystals were obtained from the 60 mg. of material. This sample (14 mg.) was recrystallized from 1:1 (v/v) acetone-water and then had m.p. $144\text{--}146^\circ$. It was shown by the following evidence to be androst-16-en-3 α -ol as isolated in the previous experiment. Some of this material was sent, at his request, to Dr K. Dobriner of the Sloan-Kettering Institute for Cancer Research, New York, for determination of its infrared absorption spectrum. The curves (sent to us by Dr Dobriner) obtained with our material and that isolated from swine testes by Prelog & Ruzicka (1944) were identical.

The crystalline fractions 10, 11 and 12 (6.7 mg.) were combined and recrystallized from methanol containing a little water. The crystals melted at $224\text{--}226^\circ$; mixed melting point with pregnane-3 α :20 α -diol, $225\text{--}229^\circ$.

Enzymic hydrolysis experiments. These were carried out with the rat-liver preparation of Talbot, Ryan & Wolfe (1943). With this preparation (4 hr. at pH 5 in acetate buffer), *NaPG(V)* (50 mg.) gave pregnane-3 α :20 α -diol (17 mg.). Crude *PLG* (221 mg.) containing 115 mg. (as *NaPG*) of glucuronide gave about 10 mg. of crude androst-16-en-3 α -ol, m.p. approx. 130° , with the characteristic smell. Further purification was made difficult by fatty material derived from the glucuronidase preparation.

Table 3. *Chromatogram of hydrolysed PLG from male urine*(60 mg. digitonin non-precipitable material on 3 g. Al_2O_3 ; see text.)

Fraction	Eluted with		Eluate	
	Vol. (ml.)	Solvent	Wt. (mg.)	Appearance
1	10	70 % (v/v) benzene/light petroleum	11.1	Gummy
2	10	70 % (v/v) benzene/light petroleum	7.1	Crystalline, musk smell
3	10	70 % (v/v) benzene/light petroleum	7.1	Crystalline, musk smell
4	10	70 % (v/v) benzene/light petroleum	3.2	Crystalline, musk smell
5	10	70 % (v/v) benzene/light petroleum	1.0	Crystalline, musk smell
6	10	80 % (v/v) benzene/light petroleum	Nil	—
	10	90 % (v/v) benzene/light petroleum	0.9	Gum
7	10	Benzene	Nil	—
	10	10 % (v/v) ether/benzene	1.1	Gum
8	10	30 % (v/v) ether/benzene	Nil	—
	10	50 % (v/v) ether/benzene	1.5	Gum
9	10	70 % (v/v) ether/benzene	Trace	—
10	10	Ether	0.5	White, crystalline
11	30	Ether	4.2	White, crystalline
12	20	Ether	2.0	White, crystalline
13	40	Ether	Trace	—
14	20	10 % (v/v) ethanol/ether	5.2	White, amorphous
	20	20 % (v/v) ethanol/ether	—	—
	20	30 % (v/v) ethanol/ether	Nil	—
15	20	50 % (v/v) ethanol/ether	Nil	—
	20	70 % (v/v) ethanol/ether	Nil	—
	20	Ethanol	11.8	White, amorphous
16	10	Ethanol	Nil	—
	20	Ethanol	Trace	—
	20	Ethanol	1.5	White, amorphous
	20	Ethanol	—	—
17	20	Ethanol	Trace	—
Total			58.2 mg.	

Hydrolysis of PLG from women's urine. The PLG from 61 l. of the urine of women in the 'follicular' phase of the menstrual cycle amounted to 215 mg. of glucuronide contained in material of 69% purity (see p. 38). The whole (311 mg.) was dissolved in 40 ml. of water, made up to 400 ml. with Lablemco peptone broth, seeded with the same strain of *Staph. albus* as used in the experiments described above and incubated for 6 days at 37°. The mixture was sterilized by heating for 20 min. at 95°, and filtered. The filter paper and filtrate were extracted with ether, but the combined ether residue weighed only 78 mg.

The ether-soluble material, partially dissolved in benzene, was put directly on to a column of Al_2O_3 (0.5 g.). Only the first fraction, which weighed 14.0 mg. and was eluted with 15 ml. of benzene, was investigated. It was semi-crystalline and smelt strongly of musk. This material was twice crystallized from dilute acetone and then melted at 149–150°, with previous softening. Recrystallization from about 1 ml. of light petroleum gave small white crystals which melted sharply at 160–161° and still smelt strongly. Yield, <1 mg. Material of similar melting point and properties was obtained by purification (sublimation, crystallization, chromatography) of the residue left on evaporation of the dilute acetone mother liquors.

RESULTS

Preliminary purification of PLG. Table 4 gives the yield of PLG at various stages in the extraction procedure as finally adopted. The figures for ethanol-soluble and -insoluble material refer to the yields obtained when the final butanol residue (see p. 37), instead of being taken up in methanol, was extracted several times with ethanol and the residual material then taken up in water or methanol. Batches *L* and *M* (combined) and subsequent batches were not fractionated in this way. These figures show some variation, but the average yields were satisfactory. The overall recovery of 85.5% of the PLG from the barium phosphate precipitate may be noted. The loss of NaPG by butanol extraction of pure aqueous solutions was about 16% (Bisset *et al.* 1948), and it is likely that the loss of PLG at this stage was at least of the same order; therefore, losses due to hydrolysis or any other cause were probably negligible. The average yield, in fifteen experiments, of PLG (as obtained on barium phosphate under the earlier

Table 4. *Yields of PLG from men's urine at various stages in the extraction procedure*

(For details of procedure, see p. 37. All weights and percentages of glucuronide in PLG are expressed as NaPG.)

Urine (batch of 20 l.)	PLG in butanol residue (mg.)	Wt. of Ba phosphate ppt. (g.)	PLG entrained on Ba phosphate ppt. (mg.)	Fraction	Acetone-insol. PLG (final residue) from batches of Ba phosphate ppt.		Percentage of entrained PLG recovered as acetone- insol. final residue
					PLG assay (mg.)	Purity (%)	
A	76.8	10.91	63.2	—	No systematic figures available as these lots were pooled and used in explor- atory extraction experiments		
B	82.4	11.34	72.7	—			
C	75.0	10.97	52.3	—			
D	82.9	11.24	69.8	—			
E	90.0	13.06	78.3	—			
F	80.1	11.45	70.1	{ Ethanol-sol.	88.7	50.2	79.2
G	81.8	10.90	71.7	{ Ethanol-insol.	23.6	18.9	
H	—	11.14	51.3	{ Ethanol-sol.	53.7	48.6	70.8
I	—	11.54	66.5	{ Ethanol-insol.	29.7	21.6	
J	—	11.49	98.9	{ Ethanol-sol.	112.3	52.7	78.1
K	—	11.73	76.1	{ Ethanol-insol.	24.3	19.0	
L	—	11.35	89.4	Ethanol + water sol.	139.2	35.0	91.3
M	—	11.46	63.1				
N	—	11.83	93.4	Methanol-sol.	195.4	51.2	99.8
O	—	11.58	102.5				
P	—	11.43	122.9	Methanol-sol.	180.5	53.7	83.7
Q	—	11.78	92.7				
R	—	11.64	95.3	Methanol-sol.	157.3	50.7	88.4
S	—	10.06	82.5				
Mean yields/l. of urine	4.1	0.57	4.0		3.6		

Table 5. *Recovery of PLG from men's urine on alumina columns*(1 g. Al₂O₃ used except where stated otherwise.)

Column	Material put on		Material recovered		PLG assaying above 69% (as NaPG)	
	Total solid (mg.)	Total PLG (as mg. NaPG)	Wt. PLG recovered (as mg. NaPG)	Percentage PLG recovered	Total wt. (mg.)	Average assay of samples (%)
1	101.6	54	26	48	17.5	72
2	103.3	53	48	91	36.3	90
3	99.8	51	43	84	36.6	73
*4	300.7	153	97	63	—	—
5	100.0	51	40	78	133.2	69
6	99.2	51	40	78		
7	99.1	51	41	80		
8	300.8	119	107	90	—	—
9						
10						
11	98.4	55	56	102	62.3	69
12						
13	199.8	111	86	77	115.1	75
14	309.9	163	133	82	151.2	71
15						
16						
*NaPG(V)	100.5	98	Approx. 22	Approx. 22	—	—

* 10 g. Al₂O₃.

'dilute' entrainment conditions) from 1 l. of men's urine extracted thoroughly with full quantities of fresh butanol, was 6.4 mg. Our time-saving procedure of extracting five 4 l. lots of urine with the same single lot of butanol (2 l.) gave an average yield of 4.0 mg./l. at the same stage (Table 4), and therefore entailed a loss of about 38 % of the total extractable *PLG*.

Purification of PLG on alumina columns. The results obtained on all but the first, exploratory, column are summarized in Table 5. Since the loss of *PLG* on column 4 was large, and no high-assay glucuronide was obtained from it, the use of only 1 g. of Al_2O_3 for approx. 100 mg. of *PLG* was continued in later experiments; the last three groups of columns are bracketed together as they were treated simultaneously in parallel. Column 11 was a separation of the main middle fraction (assaying at approx. 60 % as NaPG) from columns 8–10. The NaPG(V) column was a failure for no reason that was ascertained.

These results indicate that an average of 79 % of *PLG* put on the columns was recovered. It was apparent that no clear-cut separation of the components of *PLG* could be attained, particularly as a second attempted separation failed to effect more than a slight further purification of the glucuronide material.

Nature of PLG from men's urine. This clearly contained androst-16-en-3 α -ol (I) or some substance which was converted into it under very mild conditions, and also pregnane-3 α :20 α -diol. Small amounts of ketonic material and of digitonin-precipitable substances were present.

DISCUSSION

Men's urine

Since the androstenol was isolated without the use of bacteria for hydrolysis, it was presumably not a bacterial artefact. The high concentration of glucuronide in the purer specimens of *PLG*, together with the absence of smell in these specimens, made it appear very probable that the androstenol was, in fact, present as a glucuronide in the original urine, although *PLG* was a hygroscopic gum and no crystalline glucuronide could be isolated.

It is much more difficult to answer the questions: (i) what proportion of *PLG* is the glucuronide of androst-16-en-3 α -ol? (ii) to what extent is the androstenol estimated in a quantitative *PLG* determination? There is no doubt in our minds that the chief constituent of the purified *PLG* from the men's urine which we have handled was in fact the androstenylglucuronide, and indeed the systematic examination of hydrolysed *PLG* resulted in the isolation of only one other crystalline substance (pregnanediol) in small amount. However, the hygroscopic nature of *PLG* and the difficulty of reproducing samples of defined purity (as measured by the glucuronide assays) make it impossible to say more than that it is our belief that perhaps 50 % or more of our purest *PLG* probably consisted of sodium androst-16-en-3 α -ylglucuronide. An answer to the second question involves a consideration of the degree to which the larger-scale preparation of *PLG* reproduces the conditions of the quantitative assay previously described, together with some assessment of the extent to which this assay estimated the androstenylglucuronide. A comparison (see p. 37) of the quantitative assay with the preparative process now described shows that in the latter large losses of glucuronide occurred on entrainment, etc., although entrainment was in fact carried out from more concentrated urine extracts than were used in the quantitative *PLG* estimation. Thus the preparative process was at best only very roughly a reproduction on a larger scale of the assay, and deductions from it apply only to a limited extent to *PLG* estimations. Furthermore, the losses incurred on entrainment of the androstenylglucuronide are quite unknown and could not be estimated until a pure sample of this substance is available.

It therefore seems impossible to say more than that, since androst-16-en-3 α -ylglucuronide is a major constituent of *PLG* from men's urine, and since the solubilities of this latter mixture are undoubtedly very similar to those of pregnanediol-glucuronide, a *PLG* estimation in male urine does include a substantial contribution from the androstenylglucuronide present.

Women's urine

It was not possible, on the basis of our results, to say that the female 'follicular' urine examined by us contained androst-16-en-3 α -ol, although the strong musk-like smell of the appropriate fractions led us to suspect that this was the case. However, the crystalline material of m.p. 160–161° isolated still smelt strongly. Further purification of larger amounts of this material ought to enable one to decide whether it is a new musk-smelling substance or whether (as seems more probable) the smell was associated with traces of the androstenol present as an impurity.

In view of the previous isolation (Prelog & Ruzicka, 1944) of androst-16-en-3 α -ol from swine testes, it may perhaps be tentatively assumed that this compound is also of testicular origin in the human male. If this is the case, further consideration will be needed concerning the origin of the musk-smelling material undoubtedly present in a conjugated form in women's urine.

SUMMARY

1. 'Pregnanediol-like-glucuronide' (PLG), isolated by a larger-scale modification of a previously described analytical procedure, has been purified and hydrolysed by use of (a) a strain of *Staphylococcus albus*, and (b) a crude glucuronidase preparation.

2. PLG from men's urine yielded, after bacterial hydrolysis, a little ketonic material, a little digitonin-

precipitable material, a little pregnane-3 α :20 α -diol and, as chief product, crystalline androst-16-en-3 α -ol. The androstenol was also isolated after enzymic hydrolysis.

3. PLG from women's 'follicular' urine yielded, in a preliminary experiment, a musk-smelling fraction from which material of melting point about 160° was obtained. It was not possible to prove the presence of the above androstenol.

4. The significance of these results is discussed.

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Some Observations on the Kinetics of Haemoglobin in Solution and in the Red Blood Corpuscle

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The kinetics of the rapid reactions of oxygen and carbon monoxide with the oxygen-carrying pigments have been studied by the rapid-flow methods of Hartridge & Roughton (1923*a-c*, 1925, 1927), Millikan (1932, 1933*a, b*, 1936) and Roughton (1934*a-d*). For myoglobin (Mgb), Millikan found that the rate of combination of oxygen could be expressed by the equation

$$\frac{d[\text{O}_2\text{Mgb}]}{dt} = k'[\text{O}_2][\text{Mgb}] - k[\text{O}_2\text{Mgb}], \quad (1)$$

and furthermore that $k/k' = K$, the equilibrium constant of the reaction ($K = [\text{O}_2][\text{Mgb}]/[\text{O}_2\text{Mgb}]$) as determined from the dissociation curve, which is in this case a rectangular hyperbola. These simple relations are to be expected on the basis of the Law of Mass Action, since the myoglobin molecule only contains one iron atom and thus reacts reversibly with one molecule of oxygen according to the equation