The Estimation of a Fraction Resembling Pregnanediol Glucuronide in Human Urine

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The amount of pregnanediol in human urine can be estimated by an assay of the (partially purified) sodium pregnanediol glucuronide (NaPG) extracted by butanol (Venning, 1937, 1938), or of the pregnanediol itself, extracted from the urine after hydrolytic treatment (Astwood & Jones, 1941; Talbot, Berman, MacLachlan & Wolfe, 1941). The latter method is capable of considerable specificity and sensitivity and has been extensively developed. Jayle, Crépy & Wolf (1943) and Jayle & Libert (1946) used methods in which the glucuronide, purified after isolation from butanol extracts, was estimated by the naphthoresorcinol reaction, and Allen & Viergiver (1941) precipitated the glucuronide from butanol extracts with lead nitrate and estimated reducing substances after acid hydrolysis.

The present work arose out of an attempt to improve existing methods, and it was decided in the first place to employ the naphthoresorcinol reaction for estimating glucuronide. According to Bucher & Geschickter (1941), free pregnanediol apparently does not occur in human urine. It seemed to us that the method of Jayle *et al.* (1943) incorporated many of the known sources of error of the original Venning method, which is admittedly not suitable for very small amounts of NaPG, whilst that of Allen & Viergiver (1941) likewise seemed better adapted for assay of quantities larger than those which we wished to investigate. We have, therefore, devised a technique in which the glucuronide is 'entrained' (presumably as the barium salt, cf. Westphal, 1944) on a precipitate of barium phosphate, and is estimated after isolation in this way. This method enabled us to obtain accurate recoveries of small amounts of NaPG after butanol extraction from pure solutions. It was found, however, that when applied to urine of normal men, or of women in the follicular phase of the menstrual cycle, it indicated the presence of glucuronide, resembling NaPG closely in its solubility in butanol and its behaviour on entrainment, in great excess of the amount of NaPG which would be expected. The total glucuronide found in this fraction we have called 'pregnanediol glucuronide-like glucuronide', or *pregnanediol-like glucuronide* (PLG) (Bisset, Brooksbank & Haslewood, 1947) and we have attempted to estimate it. We have introduced a modification into the naphthoresorcinol method.

METHODS

(Including results of standardization of the method of estimating NaPG)

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). We found 1 mg. of NaPG(V) $\equiv$ 0.859 mg. NaPG(M) $\equiv$ 0.777 mg. of anhydrous sodium pregnanediol glucuronide (NaPG) (Marrian & Gough, 1946). 1 mg. NaPG $\equiv$ 0.618 mg. of pregnanediol. NaPG(V) was kept in a vacuum desiccator over H_2SO_4 until weighed out for use. Analysis indicated that, after this treatment, NaPG(V) contained about 1.5 mol. H_2O . Solutions of sodium pregnanediol glucuronide were kept at 0–5°.

Purification of butanol. *n*-Butanol (Commercial Solvents Ltd.) was distilled at 112–118° over KOH (5 g./l.). It was then redistilled at c. 70°/20 mm., with an air leak and in the presence of 1 g./l. of 4-aminophenol and 1 ml./l. of 5*N*-NaOH. It was finally redistilled at c. 60–70°/20 mm. This process removed substances sometimes present which tended to destroy NaPG when solutions with butanol were evaporated *in vacuo*.

Test of purified butanol. A volume of 250 ml., with 2 or 3 ml. of NaPG(V) solution (50 mg./100 ml.), was completely evaporated *in vacuo* as described below for urine extracts. The residue was heated to c. 90° with 10 ml. of water, the cooled solution was filtered and the glucuronide in 2 ml. of the filtrate ($\equiv$ 0.2 mg. or 0.3 mg. NaPG(V)) was entrained and estimated as described under 'entrainment of glucuronide' (below). Allowing for known losses, a recovery of not less than 95% was considered satisfactory.

Naphthoresorcinol solution. A small excess over the amount of naphthoresorcinol (Hopkin and Williams) needed for the day's experiments was weighed out and dissolved at c. 50° in water to make a 0.025*M*-solution, which was then filtered. In earlier experiments, naphthoresorcinol solution (0.025*M*) was made and 'aged' as described, e.g., by Maughan, Evelyn & Browne (1938) and Hanson, Mills & Williams (1944): this procedure is discussed below.

The naphthoresorcinol reaction

It is clear from the literature that there is little agreement as to the best method of preparing the naphthoresorcinol reagent. We have found that the amount of final extractable colour produced with a given quantity of NaPG varies with the age of the naphthoresorcinol solution used, reaching a peak after about 2 days' storage at 0–5°, and then

tending to become less: the reagent 'blank' increases (cf. Maughan *et al.* 1938). With old naphthoresorcinol solutions the final colour tends to be obviously more red. We have found also that fresh naphthoresorcinol solution may give very inconstant amounts of colour with the same amount of NaPG. This state of affairs militates seriously against the accuracy and reproducibility of the method. The facts suggest that the actual formation of coloured matter in the reaction is not in fact due to interaction of naphthoresorcinol and glucuronic acid, but rather that the effective reagent is some unstable oxidation product of naphthoresorcinol; this product, we found, is not, as appeared possible, 3-hydroxy-1:4-naphthoquinone. We therefore considered the possibility of adding to fresh naphthoresorcinol solution a weak oxidizing agent and thus effecting at once a reproducible 'ageing' process. Dr R. V. G. Ewens suggested, *inter alia*, $K_3Fe(CN)_6$ for this purpose and we found it satisfactory. One ml. of 0.05M- $K_3Fe(CN)_6$ $\equiv$ 2 ml. of naphthoresorcinol solution (0.025M). We therefore first tried the effect of adding 0.1, 0.2, 0.4, . . . 1.0 ml. of 0.05M- $K_3Fe(CN)_6$ to the reaction tubes. With 0.6–1.0 ml. $K_3Fe(CN)_6$ the final colours were increasingly red and the reagent 'blank' much increased. The use of 0.1 ml. 0.05M- $K_3Fe(CN)_6$, however, gave an appearance during the reaction very similar to that given by 'aged' naphthoresorcinol solutions, a low 'blank' and constancy of colour. We therefore used this amount, and employed fresh, filtered naphthoresorcinol solution, as described above.

Estimation of NaPG. Quantities were measured into wide test tubes (15 $\times$ 2 cm.) as follows:

Test	Blank
NaPG solution + water to make 1.4 ml.	1.4 ml. water
0.1 ml. 0.05M- $K_3Fe(CN)_6$	0.1 ml. 0.05M- $K_3Fe(CN)_6$
* { 2.0 ml. 2N-HCl	* { 2.0 ml. 2N-HCl
{ 1.5 ml. 9N-HCl	{ 1.5 ml. 9N-HCl
2.0 ml. fresh naphthoresorcinol solution (see above)	2.0 ml. fresh naphthoresorcinol solution

The contents of the tubes were then thoroughly mixed and the colour developed, extracted and estimated as described below for urine extracts.

Reproducibility. With the above method, a series of eight results with 0.15 mg. of NaPG(V) gave (colorimetric readings; Ilford 404 green filter): average E , 0.3254, σ of an individual reading, ± 0.0036 ('blank', 0.03). Eight results with 0.1 mg. of NaPG(V) as 'standard' at different times gave (difference between 'test' and 'blank' colorimeter readings; Ilford spectrum yellow filter): average E , 0.2795, σ of an individual reading, ± 0.0076 .

Proportionality. Beer's law was fairly closely obeyed between 0.05 and 0.25 mg. NaPG(V) (0.04–0.20 mg. NaPG).

* 3.5 ml. of 5N-HCl could be used: we used acid of the two strengths because we had to do so in entrainment experiments and wished to test the procedure.

Smaller amounts were not examined, and above 0.2 mg. NaPG, the colour increased only slightly. Fig. 1 shows results of a proportionality experiment.

Fig. 1. Relationship between colorimeter readings and NaPG(V) estimated. Ilford 404 green filter.

The slight deviation from proportionality at the lower values we believe to be due largely to the impossibility of controlling the heating conditions (cf. Mills, 1946). According to Hanson *et al.* (1944) hydrolysis and colour development (in approx. 4.5N-HCl) may not be complete for 4 hr. and we do not believe that the reaction in our conditions (2.5N-HCl) is complete in 2.5 hr. However, the use of the reduced time does not appear to introduce a serious error, except perhaps when an artificial standard is used (see below). Solutions found to give colorimetric readings above the figure for 0.2 mg. NaPG (0.25 mg. NaPG(V)) were suitably diluted and re-estimated.

Entrainment of glucuronide. In preliminary experiments we found that pregnanediol glucuronide could be entrained on precipitates of $BaSO_4$ or of barium phosphate and, to a lesser extent, on other precipitates. Entrainment did not occur effectively at concentrations of sodium pregnanediol glucuronide below about 5 mg./100 ml. and the process was, therefore, not directly applicable to urine; however, we decided to apply it to urine concentrates made with butanol. Barium phosphate was used as the entraining precipitate because it could be dissolved in the HCl used for the naphthoresorcinol reaction. Entrainment from 2 ml. of aqueous solutions of NaPG diluted with 2 ml. of water was carried out exactly as described for urine extracts (below): the precipitates were not washed.

Losses on entrainment. These are shown in Table 1. The results are possibly not inconsistent with the assumption that there is a loss (perhaps due to solubility) of an almost constant amount of glucuronide on entrainment. A figure which we used in later experiments (see below) was 0.05 mg. of NaPG(V).

Washing of precipitates. To test the washing procedure used for precipitates from urine extracts, glucuronide was entrained on barium phosphate as described for urine (below) and the precipitates, drained from supernatant liquid, were washed as described for those from urine extracts. Losses on washing by this method are shown in Table 2.

Table 1. *Losses on entrainment of sodium-pregnanediol glucuronide*

(Results expressed as pure NaPG.)

NaPG entrained (mg.)	Av. loss (%)	σ of an individual % loss	No. of determinations
(a) Losses on entrainment from 2 ml. of NaPG(V) solution diluted with 2 ml. of water			
0.39	10.3	± 4.4	6
0.31	15.9	± 3.2	6
0.23	14.3	± 3.4	6
0.16	20.6	± 4.2	5
0.08	37.2	± 8.3	6
(b) Losses on entrainment from 2 ml. of NaPG(M) solution diluted with 2 ml. of water			
0.36	11.2	± 3.5	6
0.18	13.9	± 2.3	6

Table 2. *Losses on washing entrained glucuronide (from NaPG(V))*

Amount entrained (as mg. NaPG)	Av. % lost on washing	σ of an individual % loss	No. of determinations
0.31	1.0	± 0.45	12
0.23	1.3	± 0.73	12
0.16	4.5	± 0.73	12
0.08	8.0	± 1.94	12

The actual losses varied between 0.003 and 0.007 mg., as NaPG.

Extraction of NaPG(V) with butanol. Portions of 4 ml. each of a solution of NaPG(V) (50 mg./100 ml.) were added to 250 ml. of water containing 5 g. zinc acetate. The mixture was extracted with butanol, and the butanol was washed and evaporated to dryness as described for urine extracts (below).

The residue was heated almost to boiling with 10 ml. of water, and the cooled extract filtered; 2 ml. of the filtrate were diluted to 4 ml. and the estimation of glucuronide (entrained but not washed) carried out as described for urine extracts (below). In two experiments losses were as follows (entrainment losses subtracted): NaPG(V) (as mg. NaPG) added to 250 ml. water, 1.55, 1.55; extraction loss, 15.5, 17.5%.

Preservation of urine. We found this most important in the estimation of PLG output (see, especially, Bucher & Geschickter, 1941). For example, in one series of 24 hr. collections, unpreserved urine, kept cold, was found to contain no measurable PLG, although the final (night) specimen had been added not more than 6 hr. before analysis. After experimenting, we finally added 3 g. of a 2:1 (w/w) mixture of finely powdered thymol and salicylic acid to the Winchester bottle in which absolutely fresh urine specimens were collected. Such urine (10 samples) showed no visible growth of micro-organisms after 48 hr. at 37°. The PLG content of preserved urine, kept at 37°, from a subject in the 9th month of pregnancy, did not fall until after 48 hr. As a routine, we kept urines, preserved as stated, at 0–5° prior to the analysis, which was in any case carried out as soon as was practicable.

Preliminary treatment of urines. To 300 ml. of urine we added 6 g. of zinc acetate. The mixture was shaken and filtered. This process made subsequent butanol extraction easier, and did not appear to affect the results of PLG determinations on urine from non-pregnant women. Without exhaustive experiments, one might hesitate to apply it to urines of very high PLG contents because of possible losses in the precipitate produced with zinc acetate: in a few experiments we have not observed such losses.

Estimation of PLG in urine. Urine (300 ml.), preserved as above, was treated with zinc acetate (6 g.), and the mixture was shaken and filtered; 250 ml. of the filtrate were mixed by rotation in a separating funnel with successive lots of 100, 50 and 50 ml. of purified butanol. Complete clearing of the lower layer was not awaited longer than c. 5 min. The combined butanol extracts were washed by rotating with 100 ml. of 0.2N-NaOH and then with two separate lots of 20 ml. of NaCl solution (2%, w/v): separation of layers was rapid and apparently complete. The butanol was now evaporated at 50–55°/c. 20 mm. and the residue finally dried by evaporation with ethanol (dried over CaO) *in vacuo*. The residue was dissolved in one of two ways: either (a) it was heated almost to boiling with (10 × 1500/x) ml. of water (where x ml. was the 24 hr. volume of the urine), or 10 ml. of water where the 24 hr. volume was unknown or unimportant; or (b) the residue was dissolved in 2 ml. of N-NaOH in the cold, followed by 2 or 3 ml. water; phenolphthalein indicator was added, and such quantities of N and then of 0.1N-HCl as were required to discharge the pink colour, water being finally added to make the final volume (10 × 1500/x) or 10 ml. as above.

The butanol residue from urine extracts contained a certain amount of acidic gums, as well as preservative, and it proved difficult to dissolve completely all the glucuronide it contained unless heating or alkali were employed. It was also very important that the final aqueous extract of the residue should not be so alkaline as to produce a precipitate when BaCl₂ was added (see below), for such a precipitate would entrain extra, and uncontrollable, amounts of glucuronide. The effect of making the final volume (10 × 1500/x) ml. was to 'correct' to a standard 24 hr. volume of 1500 ml. of urine, and so to obtain successive urine extracts in approximately the same concentration, whatever the actual 24 hr. volume: since PLG entrained depends partly on concentration (see below), we considered this manoeuvre to be important. The solution of the residue, made as above, was filtered. A 2 ml. portion of the filtrate (F), in a 15 ml. conical centrifuge tube, was mixed with 2 ml. of water. The solution was treated with 1 ml. of 0.5M-BaCl₂, followed, after it had been cooled for 10 min. in ice, by 1 ml. of Na₂HPO₄ (0.05M, containing 50 ml. of N-NaOH/litre).

The tube was stoppered, the contents well mixed and again allowed to stand for 10 min. at 0°. Acetone (0.5 ml.) was used to wash the stopper and sides of the tube, so as to form an upper layer. The tube was then centrifuged at 2300 r.p.m. (radius of centrifuge head, 16.2 cm.) for 10 min. The supernatant fluid was poured off and the tube drained by inversion on filter paper. Acetone (2 ml.) (previously distilled over CaO) was added and stirred up with the precipitate with a fine wire; the tube was centrifuged for 10 min., the supernatant fluid discarded and the precipitate drained as before. The tube was then washed out with 10 ml. of saturated barium phosphate solution (added

from a pipette *without* disturbing the precipitate), centrifuged for 5 min. and drained as before: this last washing was repeated. Finally, the precipitate was dissolved in 4 ml. of 2N-HCl. 'Blank' barium phosphate precipitates (from 4 ml. of water, then BaCl₂ and Na₂HPO₄ as above) were made and centrifuged: these were not washed; they were dissolved in 4 ml. of 2N-HCl.

Wide test tubes (15 × 2 cm.) were now prepared as follows:

Test (duplicated)	Standard (duplicated)	Blank
0.5, 1.0 or 2.0 ml. of HCl solution of ppt. and 2N-HCl to make 2 ml.	0.2 or 0.3 ml. NaPG(V) solution (50 mg./100 ml.)	—
1.4 ml. water	1.2 or 1.1 ml. water	1.4 ml. water
—	2.0 ml. 2N-HCl solution of 'blank' barium phosphate ppt. made as above	2.0 ml. 2N-HCl solution of 'blank' barium phosphate ppt. made as above

(All tubes) 1.5 ml. 9N-HCl, 0.1 ml. 0.05M-K₃Fe(CN)₆ and 2.0 ml. naphthoresorcinol reagent (above)

The contents of each tube were thoroughly mixed and small funnels were placed in the mouths of the tubes, which were suspended by rubber bands clear of the bottom of a water bath. The water was heated to 100° for 2.5 hr., the contents of each tube being again mixed shortly after heating had begun. The tubes were now cooled in ice and the contents of each in turn poured and washed into a small separating funnel with 14 ml. of ethanolic ether (20% (v/v) ethanol/peroxide-free ether). The stoppered funnel was vigorously shaken and, after separation, the aqueous portion of the contents run off. The coloured extracts were poured as quickly as possible from the mouth of the funnel into colorimeter tubes, which were immediately stoppered. The colours, which were stable for some time in absence of strong daylight (see below), were measured in a King (1946) type of single-cell photoelectric colorimeter (Gambrell Ltd.) with an Ilford 404 green or Spectrum yellow filter. The 'blank' reading was subtracted from each of the others.

Calculation. Since the standard contained 0.15 or 0.1 mg. NaPG(V), the concentration of PLG (as NaPG(V)) was reading of 'test' (minus 'blank') × 0.15 or 0.1 mg. in 0.5, 1.0 or 2.0 ml. of HCl solution of barium phosphate precipitate. Hence the amount present in 4 ml. of HCl solution, i.e. entrained on to the precipitate from a known volume of urine, was calculated. Corrections for entrainment, washing, or butanol-extraction losses could not be applied, because of ignorance of the chemical nature and physical properties of PLG. The figures, as NaPG(V), could be corrected by reference to NaPG(M) and expressed as NaPG or pregnanediol (see above).

For urines very rich in PLG, e.g. those of late pregnancy, smaller amounts could be extracted and time thus saved in butanol evaporation. For some experiments, we were able to use 30 ml. of pregnancy urine, with one-tenth of the quantities of butanol, etc., for the extraction and washing process: the results agreed with those of larger-scale experiments.

With such urines, quantities of less than 2 ml. of the filtered aqueous extract (F) of the butanol residue could be diluted to 4 ml. with water, before entrainment, or amounts of less than 0.5 ml. of HCl solution of barium phosphate precipitate could be used for the estimation.

When larger amounts than 2 ml. of the filtrate (F) were used for entrainment, much higher results for PLG were often obtained (see under RESULTS). We do not consider that the extra glucuronide thus entrained is all 'PLG'.

Use of an artificial standard. In view of the difficulty of obtaining a suitable glucuronide of known purity as a standard, an artificial standard would clearly be of great value in the naphthoresorcinol reaction. Examination of

the final coloured extract, prepared under our conditions, with a Hilger-Nutting spectrophotometer showed that the extract, provided it was kept in shadow, had almost constant absorption between 560 and 600 mμ., although its colour altered on exposure to strong daylight. It therefore seemed possible to use the grey solution of Thomson (1946) as a standard, with the Ilford Spectrum yellow light filter. Thomson's solution was diluted to twice its volume with water and in 17 separate experiments the ratio

grey solution (colorimeter readings)

0.078 mg. NaPG (colorimeter readings less 'blank')

gave, average, 1.24, σ of an individual reading, ±0.06. In 16 determinations, the ratio grey solution/0.117 mg. NaPG was, average, 0.92, σ of an individual reading, ±0.03.

For clinical use the method could probably be shortened, with some loss of accuracy, by reducing the time of heating in the naphthoresorcinol reaction to 0.5 hr. In this case some preparation of NaPG would have to be used as a standard, since the factors quoted above for Thomson's grey solution are for a 2.5 hr. heating period. We have found Beer's law to be less accurately obeyed for a 0.5 hr. heating period, so that the NaPG standard would need to approximate to the 'test' in concentration. Collection of 24 hr. urine specimens could be avoided, in individuals whose creatinine output can be assumed constant, by expressing the PLG as mg./g. of creatinine excreted (Jayle & Libert, 1946).

RESULTS

Constancy of PLG estimations. The results of four sets, of three estimations each, by the above method on different (male) urines were (in mg. NaPG/l.):

Set 1	Set 2	Set 3	Set 4
3.50	3.12	3.73	7.59
3.23	2.56	4.99	6.55
2.86	2.84	5.26	6.62
Av. 3.20	2.84	4.66	6.92

Recovery of NaPG(V) added to urine extracts. (The results given below and later, except for the female cycle, were obtained with 'aged' naphthoresorcinol,

without $K_3Fe(CN)_6$.) $NaPG(V)$ solution was added to 2 ml. of the filtered aqueous solution (F) of the butanol residues from urines, prepared as above. PLG was entrained, washed and estimated as described. Standards were entrained (not washed) amounts of 0.4 mg. of $NaPG(V)$; 0.05 mg. $NaPG(V)$ was assumed as entrainment loss (see above). Results per 2 ml. of filtered aqueous solution (F) of butanol residue, were:

$NaPG(V)$ added (mg.) (0.05 mg. entrainment loss subtracted)	PLG estimated (as mg. $NaPG(V)$)	$NaPG(V)$ recovered (mg.)	Recovery (%)
0	0.350	—	—
0.150	0.489	0.139	92.6
0.350	0.730	0.380	108.4

Recovery of $NaPG(V)$ added to urine. $NaPG(V)$ in solution (50 mg./100 ml.) was added to 300 ml. of female 'follicular' urine, preserved as described. The whole process of PLG estimation was carried out, using standards of 0.4 mg. of entrained $NaPG(V)$ as above. Results are shown in Table 3.

Table 3. *Recovery of $NaPG(V)$ added to 'follicular' urine*

$NaPG(V)$ added to 250 ml. urine (16% butanol extraction loss and 0.05 mg. entrainment loss subtracted) (mg.)	PLG found (as mg. $NaPG(V)$)	$NaPG(V)$ recovered (mg.)	Recovery (%)
0	3.06	—	—
2.20	4.54	1.48	67.2
0	1.77	—	—
2.32	3.79	2.02	87.1
0	1.85	—	—
2.25	3.78	1.93	85.8
0	2.78	—	—
0.92	3.70	0.92	100.0
0	3.33	—	—
0.84	4.37	1.04	124.0

The recovery figures given in Table 3 are insufficient to form a reliable statistical basis for calculating the error of the whole method, and, in any case, they are somewhat unreal because of the unknown (and presumably variable) properties of PLG . However, they serve to show that the method should allow the estimation, with a moderate degree of accuracy, of an increase of output of $NaPG$ of 2.5 mg./l. of urine (1.5 mg. pregnanediol/l.).

PLG in various urines. Some results are shown in Table 4.

PLG in the normal female. Fig. 2 shows the results of PLG estimation for more than a complete cycle in

a woman (21 yr., recently married, nulliparous). Portions of 2 ml. of filtered extract (F) were used for entrainment.

Table 4. *PLG in human urine*

(x =ml. of filtered aqueous extract (F), of the final butanol residue used for entrainment.)

Urine	PLG (mg. $NaPG/24$ hr.)	
	$x=2$	$x=4$
Pregnancy (9th month)	41.1 38.1	56.2 66.6
Normal male	7.0 6.0 4.7 5.0	8.2 13.2 12.5 14.3
Infant female (2.5 years)	1.8	2.6

Fig. 2. *PLG in female cycle.*

All the above figures represent *minimal* values for PLG , because, as pointed out above, losses on butanol extraction and on entrainment cannot at present be estimated (except for $NaPG$) in the absence of precise knowledge of the properties of PLG .

DISCUSSION

The above results show that the method described will estimate $NaPG$ with some accuracy at concentrations of about 2.5 mg./l. in urine in the presence of the rest of the PLG complex. The method, therefore, should be useful in the study of the human menstrual cycle, the 'luteal' phase of which we have had no difficulty in detecting, presumably because of the increased output of $NaPG$ at this time. The method should also provide a rapid means of estimating PLG in late pregnancy, with small amounts of urine: further work would be needed to assess its value in early pregnancy. The conditions, other than pregnancy, in which PLG output may be increased include some forms of adrenal hypertrophy (cf. Venning, Weil & Browne, 1939; Salmon, Geist & Salmon, 1941) and, indeed, any condition in which

excretion of steroids conjugated with glucuronic acid is augmented.

With regard to the use of the term 'pregnanediol-like glucuronide (PLG)', it must be emphasized that this is a very vague conception at present, and can only be given precise significance when the results of investigations into the chemical nature of urinary glucuronide are available. At present, the most that can be said is that PLG presumably contains at least part of the constituents of NaPG(V), which may include the sodium glucuronides of pregnane-3(α)-ol-20-one (Marrian & Gough, 1946) and of pregnane-3(α):17:20-triol (Mason & Kepler, 1945) as well as of pregnane-3(α):20(α)-diol itself. It is of course possible that PLG may be found to include glucuronides not of a steroid nature, although this would be surprising in view of its solubilities. It is certainly clear that PLG from urine from normal males may contain very little NaPG. According to Westphal (1944), the amount of pregnanediol is about 0.7 mg./l. (as barium pregnanediol glucuronide), and we, ourselves, have found only traces in our PLG complex from this source.

One can vary the fraction of PLG isolated by altering the conditions of extraction and especially of entrainment: with sufficiently 'drastic' entrainment conditions one presumably includes much more soluble glucuronides, and possibly even barium glucuronate itself, in the estimations. With our conditions, however, we are of the opinion that the PLG estimated closely resembles NaPG in its solubilities and preliminary isolation experiments have shown that it gives a considerable ether-soluble fraction after hydrolysis. We were especially interested in the work of Jayle & Libert (1946), who have isolated 'steroid glucuronides' by two entirely different

methods and estimated them by the naphthoresorcinol reaction. Their results resemble our own quite closely and Jayle & Libert appear also to be dealing with glucuronide fractions similar to that which we call 'PLG'.

Finally, it may be pointed out that our entrained material may prove to contain other conjugates (e.g. organic sulphates) from urine, and may be useful for isolation of such substances.

The chemical nature of the glucuronide fraction is being investigated, but in the meantime the method is reported in the hope that it may be of value in the examination of urinary output of glucuronide.

SUMMARY

1. A method of estimating pregnanediol glucuronide by entrainment on barium phosphate and subsequent application of a modified naphthoresorcinol reaction has been developed.

2. The method when applied to urine gives analytical figures for a glucuronide fraction which we have called 'pregnanediol-like glucuronide (PLG)'.

3. Minimal figures for PLG (as NaPG) in various urines, including those of the human menstrual cycle, are reported.

4. The nature of PLG is discussed.

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