The Extraction and Hydrolysis of Steroid Monoglucuronides

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(Received 21 February 1966)

1. A method in use for the extraction of urinary steroid conjugates has been applied to study the recovery of synthetic steroid monoglucuronides from aqueous solution. 2. In the presence of dissolved ammonium sulphate (50g./100ml.), ether-ethanol (3:1, v/v, 3 × 0.5 vol.) extracted the monoglucuronides of steroids of the C₁₈, C₁₉ and C₂₁ series, quantitatively at values pH 2–9. 3. The hydrolysis of the synthetic steroid monoglucuronides by β -glucuronidase (*Patella vulgata*) has been examined with reference to the pH value of the medium, enzyme concentration and substrate concentration. 4. The rate of hydrolysis of steroid monoglucuronides was dependent upon steroid structure and upon site of conjugation. 5. The rate of hydrolysis of the monoglucuronides decreased in the order C-3 (phenolic) > C-3 β > C-17 β > C-3 α .

Endogenous and exogenous steroid hormones are excreted in the urine as free compounds and as water-soluble conjugates. There are conflicting opinions about the relative importance of the free and conjugate fractions but standard methods for the determination of urinary steroids determine only the latter, the major fraction, and neglect the small amount of free steroid present. Conventional urinary steroid analysis is based upon preliminary hydrolysis of the hydrophilic conjugates to lipophilic free steroids which can then be separated from other water-soluble urine compounds by extraction. This procedure, although simple to carry out, may not be entirely satisfactory, for there is no guarantee that the hydrolysis, whether carried out enzymically or by solvolysis (Burstain & Lieberman, 1958), is quantitative. A maximum yield of free steroid obtained by any procedure may correspond to an equilibrium position.

An additional source of uncertainty arises when an attempt is made to extract the steroid conjugates before hydrolysis. This step may be desirable to economise on enzyme supplies or to remove β -glucuronidase inhibitors which have been reported to be present in urine (Levy, 1956) or it may be essential, as when the unhydrolysed steroid conjugates themselves are studied. In the course of studies designed to establish the structure of urinary steroid conjugates (Foggitt & Kellie, 1964; Elce, Carpenter & Kellie, 1964), a unique collection of synthetic steroid monoglucuronides became available which permitted the present investigation.

The method of conjugate extraction studied was that originally described by Kamil, Smith & Williams (1953) and applied by Edwards, Kellie &

Wade (1953) and others for the extraction of urinary steroid conjugates. The β -glucuronidase preparation employed was obtained from the digestive hump of the limpet (*Patella vulgata*) by the method of Dodgson & Spencer (1953). Aspects of this problem have been studied by others. Wakabayashi & Fishman (1961) compared the ability of different β -glucuronidase preparations to hydrolyse synthetic steroid glucuronides. Modifications of the conjugate extraction procedure have been suggested by Kornel (1963) and by DePaoli, Nishizawa & Eik-Nes (1963).

METHODS

Conditions of conjugate extraction. (a) From aqueous solution. A series of stock (NH₄)₂SO₄ solutions (50g./100ml.) was prepared by titration with 9N-HCl or with 6N-NaOH to known values between pH 1 and 9. From each stock solution 10 ml. was transferred to a series of stoppered tubes (B19) and the steroid conjugate studied was introduced as a small volume of concentrated aqueous or ethanol solution. Extraction was carried out by shaking successively three times with 5 ml. of ether-ethanol (3:1, v/v). Each extract in turn was removed by Pasteur pipette and combined by filtration through Whatman no. 1 paper into a second stoppered tube. The extract was evaporated to dryness and the residue was dissolved in ethanol. Determinations were made on portions removed from this solution without preliminary hydrolysis.

Oestrogen monoglucuronides were determined directly by the Kober reagent as modified by Nocke (1961). 17-Oxo steroid glucuronides were measured by Zimmermann reaction and tetrahydrocortisone 3 α -glucuronide by blue tetrazolium [3,3'-dianisole-bis-4,4'-(3,5-diphenyl)tetrazolium chloride] with the method described by Chen, Wheeler & Tewell (1957).

(b) From urine. Ammonium sulphate (50 g./100 ml.) was dissolved in the urine and the pH was adjusted. The operations were carried out in this order and not, as previously in the reverse order (Foggitt & Kellie, 1964; Kornel, 1963; DePaoli *et al.* 1963), since the addition of the salt modifies the hydrogen ion concentration and it is impossible to record at what pH value extraction takes place. There is little advantage in adding excess of $(\text{NH}_4)_2\text{SO}_4$ to the urine (DePaoli *et al.* 1963), as the passage of ethanol into the aqueous layer during successive extractions with ether-ethanol eventually precipitates the excess. It is important to remove the aqueous layer, with its high content of $(\text{NH}_4)_2\text{SO}_4$, completely from the ether-ethanol layer. Any contamination of the organic phase with aqueous phase would add greatly to the dry weight of the extract and the sulphate might inhibit subsequent hydrolysis with β -glucuronidase. When correctly carried out the ether-ethanol procedure should yield an extract which does not give a precipitate with BaCl_2 reagent.

Conditions of enzyme hydrolysis. Study of the effect of pH on hydrolysis was carried out in 0.2M- Na_2HPO_4 -0.1M-citric acid buffer (McIlvaine, 1921), between pH 2.2 and 8.0. The steroid conjugate studied was transferred in solution to a series of stoppered tubes (B19) and the dry residue was dissolved in buffer (2 ml.) of appropriate pH value. β -Glucuronidase (Powder B/500 000 units/g.; Dodgson & Spencer, 1953) in aqueous solution (0.1 ml.) was added and the incubation was carried out at 37° for 1 hr. Substrate concentration of the C_{18} conjugates was the steroid equivalent of 12.5 $\mu\text{g./ml.}$ and for the C_{19} and C_{20} conjugates ranged between 50 and 100 $\mu\text{g./ml.}$ Because of the different rates of hydrolysis of the substrates, the enzyme concentration was adjusted (5–100 units/ml.) to give approx. 40% hydrolysis after 1 hr. The hydrolysis was stopped by the addition of conc. HCl (0.2 ml.) and water (2.0 ml.) was added. The free steroids were extracted and determined as described above.

Studies of the percentage hydrolysis of the various substrates against time, or against enzyme concentration at fixed time, were carried out in 0.5M-acetate buffer (2.0 ml.) at the optimum pH determined for each monoglucuronide. In each case hydrolysis was stopped by the addition of acid and the product of enzyme hydrolysis was extracted and determined.

RESULTS

Extraction of conjugates. Typical graphs of the effect of pH value on the extraction of steroid conjugates from aqueous solution are shown in Fig. 1. The data, which are representative of a large number of compounds examined, illustrate that for the C_{18} and C_{19} steroid monoglucuronides within the limits pH 2–8, extraction is almost quantitative. Compounds in the C_{18} steroid monoglucuronide group included oestrone 3-glucuronide, oestradiol 3-glucuronide and oestradiol 17 β -glucuronide and the results indicated that the presence of a free phenolic group had little effect on extraction. Relatively high concentrations of the oestradiol monoglucuronides added to urine (75 $\mu\text{g./ml.}$) were also satisfactorily recovered (> 90%) by extraction between pH 2 and 9.

Fig. 1. Extraction of steroid glucuronides from ammonium sulphate soln. (50%, w/v) with ether-ethanol (3:1, v/v) at various pH values. Oestradiol 3-glucuronide (●); oestradiol 17 β -glucuronide (○); testosterone glucuronide (□); tetrahydrocortisone 3 α -glucuronide (■); dehydroepiandrosterone glucuronide (Δ).

Compounds in the C_{19} steroid monoglucuronide group, included the C-3 monoglucuronides of epiandrosterone (3 β -hydroxy-5 α -androstane-17-one), dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one), androsterone, aetiocholanolone (3 α -hydroxy-5 β -androstane-17-one), 11 β -hydroxy-aetiocholanolone (3 α , 11 β -dihydroxy-5 β -androstane-17-one), 11-oxo-aetiocholanolone (3 α -hydroxy-5 β -androstane-11,17-dione) and the C-17 β monoglucuronide of testosterone.

Only with the 3 α -glucuronide of tetrahydrocortisone (3 α , 17 α , 21-trihydroxy-5 β -pregnane-11,20-dione) above pH 5 did extraction lead to loss of material and this loss can be confidently attributed to the destruction of the alkali-sensitive α -ketol side-chain of this compound. Below pH 5 the recovery of the glucuronide as measured by blue tetrazolium reagent was satisfactory (cf. Barlow & Kellie, 1959).

Alternative extraction solvents. From a study of procedures for the extraction of conjugated Porter-Silber chromagens, tetrahydrocortisone monoglucuronide, tetrahydrocortisol disulphate or the metabolites from urine of administered [4- ^{14}C]-cortisol, Kornel (1963) concluded that provided that ammonium sulphate (50 g./100 ml.) was present and the aqueous phase was adjusted to pH 1, alternative solvents could be used. A single extraction with butan-1-ol (1 vol.) or with ether-ethanol (3:1, v/v) (4 vol.) gave a high yield with all corticosteroid conjugates; a single extraction with ethyl acetate (5 vol.) removed all of the tetrahydrocortisone glucuronide but none of the tetrahydrocortisol disulphate.

Table 1. Comparison of extraction of steroid monoglucuronides from urine with various solvents

Steroid conjugate added	Ether-ethanol (3:1, v/v)				Ethyl acetate				Tetrahydrofuran			
	Extraction pH	Residue (mg.)	Glucuronide recovered (mg.)	Ratio*	Residue (mg.)	Glucuronide recovered (mg.)	Ratio*	Residue (mg.)	Glucuronide recovered (mg.)	Ratio*	Residue (mg.)	Glucuronide recovered (mg.)
Dehydroepiandrosterone β -glucuronide (Na)	5.9	159	2.4	67:1	163	2.0	82:1	374	1.95	192:1	374	1.95
	2 mg.†	159	2.4	67:1	163	2.0	82:1	374	1.95	192:1	374	1.95
	2 mg.	136	2.0	68:1	108	2.0	54:1	434	2.0	217:1	434	2.0
Oestradiol 17 β -glucuronide (Na)	6.5	74	1.98	37:1	38	0.24	158:1	317	1.6	198:1	317	1.6
	2 mg.	74	1.98	37:1	38	0.24	158:1	317	1.6	198:1	317	1.6
	2 mg.	153	2.0	76:1	124	2.0	62:1	482	2.0	241:1	482	2.0

* Ratio = Wt. of residues/wt. of glucuronide recovered.

† Endogenous steroid not taken into account.

DePaoli *et al.* (1963), describing the extraction of conjugated 17-oxo steroids, recommended the use of ethyl acetate or tetrahydrofuran and observed that the weight of dry residue obtained with ethyl acetate was about 50% of that obtained by the ether-ethanol method; the corresponding residue when tetrahydrofuran was used was claimed to be 10% of the ether-ethanol method. Data obtained in the present study do not bear this out.

Urine samples (150ml.) containing ammonium sulphate (50g./100ml.) were modified by the addition of steroid glucuronides. The pH value was adjusted and a sample subdivided (3×50 ml.). One portion was extracted with ether-ethanol (3:1, v/v) (3×25 ml.), a second was extracted with ethyl acetate (1×150 ml.) and the third with tetrahydrofuran (1×150 ml.). The extracts were separately evaporated to dryness, weighed and hydrolysed with β -glucuronidase (500 units/ml. at 37° for 16 hr.). The respective extracts were prepared by conventional procedures and were determined for steroid conjugate content. Endogenous steroids were determined separately. A comparison of the three extracting solvents has been made with reference to weight of residue and extraction of conjugate (Table 1).

These results confirm the value of ethyl acetate as a solvent for the extraction of steroid glucuronides from aqueous media at acid pH in the presence of

Fig. 2. Optimum pH values for the activity of β -glucuronidase (*Patella vulgata*) against steroid monoglucuronides. Dehydroepiandrosterone glucuronide ($\circ$); oestradiol 3-glucuronide ($\bullet$); testosterone glucuronide ($\blacktriangle$); oestradiol 17 β -glucuronide ($\triangle$); tetrahydrocortisone 3 α -glucuronide ($\square$).

dissolved ammonium sulphate. They do not confirm the low residue weight claimed when using tetrahydrofurane. Not only were heavier residues obtained when this solvent was used but much longer periods of incubation with β -glucuronidase were necessary to achieve maximum hydrolysis.

Effect of pH on the activity of the enzyme preparation. The optimum pH values of the enzyme preparation for all substrates lay between pH 4 and 5 and for most of the compounds between pH 4.1 and 4.2 (Fig. 2). Two 11-oxy-17-oxosteroid-3 α -glucuronides examined, conjugates of 11-oxo-aetiocholanolone and 11 β -hydroxyaetiocholanolone, showed a maximum at pH 4.75 but the peak of activity was broad and both were extensively hydrolysed at pH 4.2.

Specificity of enzyme preparation. Marked differences were observed in the rates of hydrolysis of the steroid monoglucuronides available for study (Fig. 3). Of all the conjugates examined, oestrogen glucuronides conjugated at the phenolic (C-3) hydroxyl group were most readily attacked. Hydrolysis of these C₁₈ steroid conjugates was markedly faster than that of the cognate compounds oestradiol 17 β -glucuronide and oestriol 17 β -glucuronide (Fig. 4).

In the C₁₉ series little difference was found in the rate of hydrolysis of dehydroepiandrosterone 3 β -glucuronide (3 β -hydroxy-5-en) and epiandro-

sterone 3 β -glucuronide (3 β -hydroxy-5 α), both of which were hydrolysed more readily than testosterone 17 β -glucuronide. The 17-oxo steroid glucuronides, androsterone 3 α -glucuronide (3 α -hydroxy-5 α) and aetiocholanolone 3 α -glucuronide (3 α -hydroxy-5 β), which are found in relatively large amounts in normal urine, were the least readily hydrolysed of the conjugates examined (Fig. 5). That it is possible to bring about complete hydro-

Fig. 3. Influence of structure on the rate of hydrolysis of steroid monoglucuronides by β -glucuronidase (*Patella vulgata*). Oestrone 3-glucuronide (●); dehydroepiandrosterone glucuronide (○); testosterone glucuronide (▲); oestradiol 17 β -glucuronide (△); tetrahydrocortisone 3 α -glucuronide (□); androsterone glucuronide (■). Steroid concentration 200 μ g./ml. and β -glucuronidase concentration 50 units/ml. Incubations were performed at 37°.

Fig. 4. Rate of hydrolysis of oestrogen (C₁₈) monoglucuronides: (a) against time (steroid concentration 12.5 μ g./ml. and β -glucuronidase concentration 250 units/ml.); (b) against β -glucuronidase concentration (steroid concentration 12.5 μ g./ml. and hydrolysis time 1 hr.). Incubations were performed at 37°. Oestrone 3-glucuronide (●); oestradiol 17 β -glucuronide (□); oestriol 17 β -glucuronide (○).

Fig. 5. Rate of hydrolysis of C₁₉ monoglucuronides: (a) against time (steroid concentration 200 µg./ml. and β-glucuronidase concentration 50 units/ml.); (b) against β-glucuronidase concentration (steroid concentration 200 µg./ml.). For solid line graphs time was 1 hr. and for broken-line graphs 16 hr. Incubations were performed at 37°. Dehydroepiandrosterone glucuronide (●); epiandrosterone glucuronide (○); aetiocholanolone glucuronide (□); androsterone glucuronide (■).

lysis of androsterone and aetiocholanolone monoglucuronides in 16 hr. (800 units of β-glucuronidase/ml.) is apparent from the graph. Complete hydrolysis of 11β-hydroxyaetiocholanolone 3α-glucuronide and of 11-oxo-aetiocholanolone 3α-glucuronide was achieved in 16 hr. at 37° with 250 units of β-glucuronidase/ml.

Only one corticosteroid metabolite, tetrahydro-

cortisone 3α-glucuronide, was available for study. Overnight incubation at 37° with an enzyme concentration of 50 units/ml. or over gave virtually complete hydrolysis (Fig. 3).

DISCUSSION

All the steroid monoglucuronides examined were extracted from aqueous solution in the presence of ammonium sulphate (50 g./100 ml.) by the ether-ethanol procedure irrespective of pH value. The exception, which must be made with tetrahydrocortisone 3α-glucuronide where loss occurs above pH 5, can be understood in light of the known instability of the α-ketol side-chain. Oestrogen and 17-oxo steroid monoglucuronides added to urine were similarly recovered and it appears that when full recovery of urinary steroid conjugates is not achieved by this extraction procedure (Edwards, 1959) that some other form of conjugation is present, or that some urinary component is interfering. The alternative use of ethyl acetate for the extraction of steroid glucuronides from ammonium sulphate solution at acid pH appears to be sound but the use of tetrahydrofuran is not advantageous.

Substrate structure had little effect on the optimum pH value for β-glucuronidase activity. In contrast, the rate of hydrolysis varied greatly not only with steroid structure [cf. glucuronides of androsterone (3α,5α) and isoandrosterone (3β,5α)] but also with the position of conjugation (cf. oestradiol 3-glucuronide and oestradiol 17β-glucuronide). The difficulty in achieving complete hydrolysis of androsterone and aetiocholanolone glucuronides is noteworthy as β-glucuronidase hydrolysis is frequently the basis for the determination of these urinary 17-oxo steroids. This structure specificity is reminiscent of that observed by Roy (1956) with a steroid sulphotase preparation obtained from the same mollusc (*Patella vulgata*); this enzyme is highly specific, hydrolysing only the 3β-sulphates of 5α- and Δ⁵-steroids and having no action on androsterone or aetiocholanolone sulphates.

The interest and support of Sir Charles Dodds, Bt., M.V.O., F.R.S., in this work is gratefully acknowledged. The author thanks Dr J. J. Schneider, Philadelphia, for the gift of a sample of tetrahydrocortisone monoglucuronide (sodium salt) and acknowledges the valuable technical assistance of Mr M. Boyce and Mr A. Tovell.

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