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The Enzymic Hydrolysis of Steroid Conjugates

2. HYDROLYSIS OF STEROID CONJUGATES IN URINE

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That urinary androgenic substances might be excreted as conjugates was first indicated by Funk, Harrow & Lewja (1929). Since that date, only two types of steroid conjugate have been isolated from urine—the glucuronides and the sulphates.

It has long been recognized that the drastic conditions of acid hydrolysis necessary to release steroids from these water-soluble derivatives in urine may result in losses or chemical transformations. Mild forms of acid hydrolysis have also been tried, but those steroids linked with glucuronic acid require for hydrolysis the stronger acid conditions which are responsible for the production of artifacts. These problems have been reviewed by Pincus (1954), and by Birke & Plantin (1954).

The successful use of bacterial glucuronidase for the hydrolysis of steroid glucuronides was reported by Buehler, Katzman, Doisy & Doisy (1949).

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Several authors (e.g. Cohen, 1951; Corcoran, Page & Duston, 1950; Cox & Marrian, 1951; Kinsella, Doisy & Glick, 1950) have since demonstrated that hydrolysis of urinary steroid conjugates with preparations of β -glucuronidase liberated considerably more formaldehyde-producing material from urine than hydrolysis and extraction at pH 1. More recently, Katzman, Straw, Buehler & Doisy (1954) have described the action of bacterial β -glucuronidase preparations on urinary oestrogen conjugates. A study of the hydrolysis of steroid conjugates by calf-spleen β -glucuronidase was made by Cohen (1951). Bitman & Cohen (1951) compared the hydrolysis of conjugated 17-oxosteroids by the acetate-buffer technique of Talbot, Ryan & Wolfe (1943), by calf-spleen β -glucuronidase and by hydrochloric acid at pH 1.

This paper describes the application of sulphatase and β -glucuronidase of molluscan origin to the hydrolysis of some urinary neutral 17-oxosteroid sulphates and glucuronides.

EXPERIMENTAL

The enzyme powders were prepared from specimens of *Patella vulgata* by the procedure described in the previous paper (Stitch, Halkerston & Hillman, 1956). The sulphatase activity of the powders used varied between 2000 and 15 000 units/g., and had an average β -glucuronidase activity of 1.5×10^6 units/g. A powder containing 9100 units of sulphatase/g. and 1.2×10^6 units of β -glucuronidase/g. was used for the study of the effect of pH, enzyme concentration, time and inhibitors on the enzymic process in urine.

Enzymic hydrolysis, extraction and estimation of urinary neutral 17-oxosteroids. In the following enzyme experiments, the urine samples were warmed to 37° in small flasks and adjusted to the required pH. An aqueous solution of the enzyme was added and the pH finally checked. The flasks were lightly stoppered with cotton wool and incubated at $37 \pm 0.1^\circ$ for the required time. The samples were then cooled and extracted four times with equal volumes of ether. The combined ether extracts were washed twice with 2N-NaOH ($\frac{1}{2}$ vol.), and finally three times with distilled water ($\frac{1}{2}$ vol.). Troublesome emulsions, when encountered, were broken by centrifuging. The washed ether extracts were dried over sodium sulphate for 3–4 hr. and filtered through a sintered-glass funnel (1A), under reduced pressure. The ether was evaporated and the residue dissolved in ethanol. Suitable portions were taken for the estimation of the 17-oxosteroids, by means of the Zimmermann reaction.

Acid hydrolysis, extraction and estimation of urinary neutral 17-oxosteroids. The urine samples were refluxed with concentrated HCl (15 ml./100 ml. of urine) and CCl_4 (20 ml./100 ml. of urine) according to the method of Callow, Callow, Emmens & Stroud (1939). The combined CCl_4 extracts were washed twice with 2N-NaOH ($\frac{1}{2}$ vol.) to remove phenolic substances, and three times with distilled water ($\frac{1}{2}$ vol.). The washed extract was dried over Na_2SO_4 for 3–4 hr., filtered through a sintered funnel and concentrated by distillation; it was then made up to 50 ml. with CCl_4 . Suitable portions were evaporated to dryness at $100\text{--}110^\circ$ (oil bath), and the 17-oxosteroid content was estimated by means of the Zimmermann reaction (Callow, Callow & Emmens, 1938).

Partition chromatography. Fractionation of the extracted 17-oxosteroids was carried out by partition chromatography on columns of silicic acid with nitromethane as stationary phase, and light petroleum (boiling range $60\text{--}80^\circ$) containing 3% of CHCl_3 as mobile phase (Jones & Stitch, 1953).

RESULTS

The effect of pH (3.55–5.5) on the rate of hydrolysis of the urinary 17-oxosteroid conjugates showed a shallow pH optimum in the region of 4.5–5.0. The rate of hydrolysis increased rapidly with increase in enzyme concentration up to about 50 mg. (approximately 450 units of sulphatase and 6.0×10^4 units of β -glucuronidase/25 ml. of urine), but further increase had little effect.

Rate of release of free 17-oxosteroids from urine by an extract of P. vulgata

The enzymic hydrolysis of 25 ml. portions of urine was studied over a period of 140 hr. After

Fig. 1. Release of 17-oxosteroids from urine by enzyme from *P. vulgata*. Portions (25 ml.) of a 24 hr. urine were incubated at $37^\circ \pm 0.1^\circ$ with 450 units of sulphatase and 6×10^4 units of β -glucuronidase in 50 mg. of powder. The pH was adjusted to 5.0 with acetate buffer. Controls without enzyme were incubated at the same pH for the same periods. Residual oxosteroid remaining after enzymic hydrolysis was determined by acid hydrolysis. The total 17-oxosteroids released by acid hydrolysis of a 25 ml. portion is shown by the height of the black area. ●—●, Limpet extract; ●---●, residual ketosteroid released by acid after enzymic hydrolysis; ○—○, untreated urine.

extraction of the 17-oxosteroids, the residual urine was hydrolysed with acid in order to obtain an estimate of the amounts of conjugated 17-oxosteroids remaining after enzymic hydrolysis. Untreated urine controls were incubated at the same pH. Results shown in Fig. 1 reveal an increase in 17-oxosteroids with time up to 70 hr., together with decreased residual 17-oxosteroid.

Removal of inhibiting ions from solution before enzymic hydrolysis of the urinary 17-oxosteroids

Sulphate. Normal male urine samples (25 ml.) were treated with 2 ml. of an aqueous solution of barium chloride saturated at room temperature (18°). The precipitated barium sulphate was left in suspension. Portions (25 ml.) of the same urine similarly treated gave no further precipitate when centrifuged and treated with additional barium chloride. The pH of each portion was adjusted to 5.0 with N-HCl, and 450 units of sulphatase and 6×10^4 units of β -glucuronidase in 50 mg. of powder were added. Incubation was carried out at 37° for periods of time varying from 5 to 96 hr. The samples were extracted and the 17-oxosteroid content was estimated by the Zimmermann reaction. Untreated urine controls were incubated at the same pH. The time-hydrolysis curves which were obtained indicated a slight acceleration of the

hydrolysis rate after precipitation of the sulphate ion (Fig. 2).

In a similar experiment on another urine sample, the precipitated barium sulphate was removed by filtration before incubation. The results again showed that the effect of sulphate removal was a slight (12%) increase in the rate of hydrolysis. Barium has been shown to be without appreciable effect on the enzymic hydrolysis of dehydroepiandrosterone sulphate and of phenolphthalein β -glucuronide (Stitch *et al.* 1956).

Sulphate and phosphate. Portions (25 ml.) of a normal male urine were adjusted to pH 11.5 with 2N-NaOH, treated with 2 ml. of a 10% aqueous solution of barium chloride and centrifuged, and the pH of the supernatant urine was adjusted to 5.0 with HCl. A total of 450 units of sulphatase and 6×10^4 units of β -glucuronidase in 50 mg. of powder was added and incubation carried out for 2 hr. at 37°. Treatment of similar urine samples with additional barium chloride after centrifuging gave no further precipitate. The results indicated a four-fold increase in the rate of hydrolysis over that of the controls which were not treated with barium chloride.

Fig. 2. Effect of removal of sulphate ion on rate of hydrolysis of urinary 17-oxosteroids. Portions (25 ml.) of 24 hr. urine were incubated at $37^{\circ} \pm 0.1^{\circ}$ with 450 units of sulphatase and 6×10^4 units of β -glucuronidase in 50 mg. of powder at pH 5.0; 25 ml. portions of the same urine were incubated, after precipitation of sulphate, under the same conditions. The total oxosteroids released by acid hydrolysis is shown by the height of the black area. The shaded area shows the untreated urine control incubated at the same pH; ●—●, effect of enzyme after precipitation of sulphate; ○---○, effect of enzyme without precipitation of sulphate; ○—○, residual oxosteroid released by acid after enzymic hydrolysis.

Table 1. Comparison of the total neutral 17-oxosteroids released by acid and by enzyme: hydrolysis of 250 ml. portions of different urines

Sample of urine	17-Oxosteroids (mg./24 hr.)		$\frac{\text{Enzyme}}{\text{Acid}} \times 100$
	Acid hydrolysis	Enzyme hydrolysis	
Normal (pooled male)	5.2*	5.0*	96.1
Mental patients (male)			
1	11.4	10.0	87.8
2	13.7	11.2	81.9
3	13.7	12.5	91.3
4	6.0	6.4	106.5
5	9.7	11.7	120.5
6	8.7	9.05	109.0
7	9.8	11.05	112.0
8	7.5	8.95	120.0
9	15.45	17.2	111.1
10	7.2	6.8	94.5
11	13.4	13.4	100.0
12	18.6	18.2	98.0
13	10.5	9.75	93.0
14	23.2	13.9	60.0
15	5.15	5.3	102.5
16	7.5	3.35	44.4
Mental patients (female)			
1	9.8	8.9	90.9
2	2.85	2.4	83.1
3	2.3	1.6	69.4
4	9.1	8.1	89.0
5	8.5	5.1	59.8
6	12.3	11.8	96.0

* mg./l.

*Effect of sulphate and phosphate precipitation
on the 17-oxosteroid content of urine*

Two 25 ml. portions of urine were treated with barium chloride as described in the previous experiment. In this case the precipitated barium salts were washed with 3 ml. portions of water and the washings added to the supernatant urine. As controls, two further 25 ml. portions of urine were treated similarly but with distilled water replacing the barium chloride solution. All four samples were then subjected to acid hydrolysis in the presence of carbon tetrachloride, and the 17-oxosteroid content of the carbon tetrachloride extracts was determined as described above. The results indicated that a significant (16%) loss of 17-oxosteroid occurred during the precipitation of the sulphate and phosphate ions. Attempts to extract the

adsorbed 17-oxosteroid from the precipitated barium salts included extraction with two 5 ml. portions of ethanol followed by acid hydrolysis (in the presence of carbon tetrachloride) of the residue. The results showed that in neither case could any 17-oxosteroid be recovered.

Comparison of acid and enzymic hydrolysis

In this series of experiments, carried out by Dr J. J. Gordon, urines from normal males and mental patients were taken for a comparison of the amounts of 17-oxosteroids released by either acid or enzymic hydrolysis.

One sample of each urine was hydrolysed with acid as described above and the second was adjusted to pH 4.5 with HCl, and an amount of enzyme containing 5000 units of sulphatase and approx. 0.75×10^6 units of β -glucuronidase was added.

Fig. 3. Partition chromatography of 17-oxosteroid extracts from normal male urines: (A) after enzymic hydrolysis; (B) after acid hydrolysis. The following steroids are eluted in the peaks: I, artifacts (chiefly 3β -chloroandro-5-en-17-one); II, unknown; III, 3:5-cycloandrostan-6-ol-17-one and androsterone; IV, androstan- 3β -ol-17-one; Va and Vb, androst-5-en- 3β -ol-17-one; testan- 3α -ol-17-one.

Incubation was carried out at 37° for 96 hr. The results of this comparison are shown in Table 1 and have been calculated in terms of androst-5-en-3 β -ol-17-one after correction for non-ketonic chromogens by the method of Cook & Rooks (1952).

Partition-chromatographic analysis of the 17-oxosteroids extracted from urine after enzymic or acid hydrolysis

Two 24 hr. specimens of normal male urine were divided equally; one portion of each specimen was subjected to acid hydrolysis and the other incubated at pH 5.0 with sufficient enzyme to give 20 000 units of sulphatase/l. of urine and approx. 3.0×10^6 units of β -glucuronidase/l. Incubation was carried out at 37° for 96 hr. After incubation the liberated steroids were extracted and washed. A portion of the total extract containing 3–4 mg. of total 17-oxosteroid was evaporated to dryness, and taken for chromatography. Fractionation was carried out as described by Jones & Stitch (1953). The chromatographic patterns obtained are illustrated in Fig. 3. These show a difference in the distribution of the peaks according to the method of hydrolysis employed.

In some cases a rather different elution pattern was obtained which seemed to indicate formation of additional polar steroids during enzymic hydrolysis.

DISCUSSION

The results described above show that the molluscan-enzyme preparations are capable of releasing some urinary neutral 17-oxosteroids from their water-soluble derivatives in urine. The comparison between the enzymic hydrolysis and the conventional acid hydrolysis shows some similarity in total 17-oxosteroid values obtained. However, on fractionation of the steroid extracts it is clear that the compounds generally accepted as artifacts of acid hydrolysis (Fig. 3, peak 1) are absent from the extract derived after enzymic hydrolysis. Furthermore, it appears that other peaks are increased when the enzyme extract is employed. It is noted that in spite of the failure of the enzyme extract to hydrolyse pure androsterone sulphate (Stitch *et al.* 1956) the chromatographic peak corresponding to the position of androsterone in the elution sequence (peak 3) was obtained in similar yield by both acid and enzymic hydrolysis. This would appear to indicate that the androsterone was not excreted, in the urine samples studied, in the sulphate form or that hydrolysis of this compound occurred by a mechanism other than that initiated by the added enzyme. Recently, Jayle & Baulieu (1952) found androsterone only in the fraction soluble in butanol

at pH 11 but not hydrolysed at pH 5.8. However, Birke & Plantin (1954) found that androsterone comprised 20% of the liberated steroids after hydrolysis of the urinary conjugates with 1:4-dioxan and chromatography and infrared spectroscopy of the hydrolysate.

Decomposition of the 17-oxosteroid by bacteria may account for the decline in steroid value observed after prolonged incubation (Fig. 1). This fall in value was also observed on butanol extracts of urine but was not a general occurrence. Attempts to prevent bacterial action by the addition of penicillin G (5000 units) and streptomycin sulphate (2.5 mg.) (without effect on the enzymic-hydrolysis rate of dehydroepiandrosterone sulphate and phenolphthalein β -glucuronide), to a butanol extract (5 ml.) of 50 ml. of urine, gave inconclusive results since control urines and urines with added antibiotic showed similar rates of hydrolysis, and neither showed the late fall in 17-oxosteroid value. A small amount of residual 17-oxosteroid always appears to remain unhydrolysed after enzymic hydrolysis has been carried out (Figs. 1, 2).

The variable results for the comparison of steroid released by acid and by enzymic hydrolysis can be accounted for by the high but variable concentration of phosphate inhibitor in urine and by difficulties in the extraction of the steroids from urine after enzymic hydrolysis.

Although this enzymic procedure has been shown to reduce the artifact normally associated with acid hydrolysis, no account has been taken of possible steroid transformations which may be effected by enzyme systems contained in the urine or in the added molluscan extracts. Such effects have been demonstrated recently (Talalay & Bobson, 1953). It is significant that microbiological hydroxylation of many steroids has recently been reported by several authors, e.g. Zaffaroni, Campillo, Cordoba & Rosenkranz (1955). Enzyme systems in the urine could have been destroyed by boiling, but this was felt to be undesirable because of the possible effects of heat on the 17-oxosteroids. The negligible blanks obtained indicated that endogenous β -glucuronidase and sulphatase were not responsible for the hydrolysis of the steroid conjugates. In view of the possibility of steroid transformation by unknown enzyme systems, the extracts obtained after this form of hydrolysis cannot necessarily be considered free from artifact.

The marked specificity of the sulphatase and the relatively high release of 17-oxosteroid from urine on occasions suggests that sulphates other than those of the 3 β -, 5 α - and Δ^5 -series may comprise a small fraction of the total 17-oxosteroids, or that hydrolysis of these conjugates may be effected by some other process.

SUMMARY

1. The application of a sulphatase and β -glucuronidase extract of molluscan origin to the hydrolysis of urinary steroid conjugates has been described.

2. The effect of varying conditions of pH, time and enzyme concentration on the rate of hydrolysis of the conjugates has been studied.

3. The influence of some urinary inorganic inhibitors on the rate of enzymic hydrolysis has been indicated.

4. A comparison of the amount of neutral 17-oxosteroids released by the methods of enzymic and acid hydrolysis has been made.

5. Partition chromatography of urinary neutral 17-oxosteroids after both enzymic and acid hydrolysis has been carried out. A difference in the distribution of various chromatographic fractions depending on the method of hydrolysis was noted.

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The Selective Solvation of the Hyaluronic Acid Complex of Ox Synovial Fluid

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This work was undertaken to test the suggestion made by Johnston (1955) that a large part of the volume occupied in solution by the coiled-chain particles of the hyaluronic acid complex (Ogston & Stanier, 1951, 1952) might be 'impenetrable' by serum albumin. Such an impenetrability could equally be regarded as a positive selective solvation of the hyaluronic acid complex by components of the solution (water and buffer salts) other than albumin (Ogston, 1954). This hypothesis was tested by studying the effect of concentration of albumin on the sedimentation of the hyaluronic acid complex in the ultracentrifuge; this is the method used by Lauffer and his colleagues (see Lauffer, Price & Petre, 1949) to estimate the 'solvation' of virus particles.

The rate of sedimentation of the hyaluronic acid complex becomes zero when the reciprocal of its effective specific volume (i.e. its effective density in solution) is equal to the density of the solution. Its effective density can be regarded as being determined by the relative proportions in the sedimenting particles of (i) unsolvated hyaluronic acid complex, (ii) solvent free of albumin, (iii) solvent containing albumin, of the same composition as that of the bulk solution.

EXPERIMENTAL

Hyaluronic acid complex. This was prepared by the method of Ogston & Stanier (1950). The complex was washed 16 times with buffer on a sintered-glass filter of average pore diameter 1μ . The resulting solution showed only a single, very sharp boundary in the ultracentrifuge, and no sign of any free protein.

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