



Further work on this rearrangement is in progress.

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### An Improved Hydrolytic Procedure for Urinary Neutral 17-Ketosteroids

RESEARCH on the excretion of urinary steroids has been handicapped by the lack of a procedure for hydrolysing steroidal conjugates without simultaneous partial destruction or transformation of the released steroids. We have therefore compared the urinary neutral 17-ketosteroids obtained by various methods of hydrolysis and extraction (for example, Talbot *et al.*<sup>1</sup>, Hamburger<sup>2</sup>, Beher and Gaebler<sup>3</sup>).

The methods were evaluated by photometric determinations of two groups of released steroids, namely, the total amount of neutral 17-ketosteroids as determined by the modification of Callow *et al.*<sup>4</sup> of Zimmermann's reaction, and the dehydroisoandrosterone fraction, a subdivision of the neutral 17-ketosteroids, as determined by the *D*-reaction (Jensen<sup>5</sup>).

Two of the seven hydrolytic procedures studied were found to be equally good. They gave a higher yield, especially of the dehydroisoandrosterone fraction, than did the other methods. In one of these two procedures use was made of simultaneous benzene extraction<sup>2</sup>; in this the urine aliquot is charged with one-tenth of its volume of 40 per cent by volume sulphuric acid and heated under a reflux condenser for 30 min. The other procedure was the routine method of this laboratory, and is as follows: to 1/200th of a 24-hr. urinary sample is added 1/10th volume of 70 per cent by volume sulphuric acid, after which the solution is heated to 100° C. for 30 min., cooled and extracted with ethyl ether.

Certain observations suggested that these two methods also involved a certain amount of destruction of steroids.

Talbot *et al.*<sup>6</sup> and Bitman and Cohen<sup>7</sup> have demonstrated that some of the neutral 17-ketosteroidal conjugates extracted from urine with *n*-butanol undergo hydrolysis when heated for 4 hr. to 100° C. in a buffer solution of pH 5.5-6.0. As to dehydroisoandrosterone, no destruction or transformation occurred.

In our investigations, aliquots of urinary specimens were adjusted to pH 3, 5, 7, 9 and 11 respectively and heated to 100° C. for 2, 4, 6, 8, 14, 30 and 48 hr. After heating for 8 hr., the steroids released from the

two above-mentioned fractions reached a maximum that persisted unchanged for a further 40 hr. heating. Variations of the pH between 3 and 11 are thus of no importance so far as some of the steroidal conjugates are concerned.

The pH of the urinary specimens received at this laboratory ranged between 4 and 10; most of them were acid. We therefore devised the following technique. Aliquots of urine were heated to 100° C. overnight and extracted with ethyl ether. The ether extracts were then purified in the usual manner and subjected to an additional washing with 2*N* sulphuric acid. The extracted urine aliquots were then hydrolysed in the routine manner described above.

Twenty urinary specimens, mostly from patients with symptoms suggesting some endocrine dysfunction, showed no determinable amounts, by ethyl ether extraction and without the use of some preparatory hydrolytic method, of compounds producing a specific colour in Zimmermann's or the *D*-reaction.

The technique using double hydrolysis gave 100-174 per cent (average 119 per cent) neutral ketosteroids as compared with the routine hydrolytic method. The first hydrolysis gave a dehydroisoandrosterone fraction of 100-168 per cent (average 148.5 per cent) as compared with the routine hydrolytic method. The steroidal fraction released in the first hydrolysis was 23.6-60 per cent (average 49.5 per cent) of the total amount of neutral 17-ketosteroids obtained by both hydrolyses.

The dehydroisoandrosterone fraction amounted to 0-96.6 per cent (average 66.0 per cent) of the steroidal fraction released by the first hydrolysis and 0-56.9 per cent (average 32.7 per cent) of the neutral 17-ketosteroids obtained by both hydrolyses.

Observations recorded in the literature of the hydrolysis of urinary conjugates of neutral 17-ketosteroids, and our own results, suggest that (a) the steroids released in the first hydrolysis are unsaturated and have been conjugated as sulphates; and (b) that the fraction of steroids producing a specific colour in the *D*-reaction consists of steroids with a double bond connected to an angular carbon atom.

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<sup>1</sup> Talbot, N. B., Butler, A. M., MacLachlan, E. A., and Jones, R. N., *J. Biol. Chem.*, **136**, 305 (1940).

<sup>2</sup> Hamburger, C., *Acta Endocrin.*, **1**, 375 (1948).

<sup>3</sup> Beher, W. T., and Gaebler, O. H., *Anal. Chem.*, **23**, 118 (1951).

<sup>4</sup> Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, **32**, 1312 (1938).

<sup>5</sup> Jensen, C. C., *Nature*, **165**, 321 (1950); *Acta Endocrin.*, **4**, 140 and 374 (1950).

<sup>6</sup> Talbot, N. B., Ryan, J., and Wolfe, J. K., *J. Biol. Chem.*, **148**, 593 (1943).

<sup>7</sup> Bitman, J., and Cohen, S. L., *J. Biol. Chem.*, **179**, 455 (1949).