The Acid-catalyzed Solvolysis of Dehydroepiandrosterone Sulfate and Its Significance in the Examination of Urinary 17-Ketosteroids*

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Burstein and Lieberman (1) have reported a procedure for the examination of urinary ketosteroid sulfates which involves the extraction of an acidified urine with ethyl acetate followed by solvolysis of the sulfates in the organic phase at 39° for 24 hours. Although the previous authors (1) recognized that these technique partitions water and mineral acid into the ethyl acetate, they did not report a detailed examination of these factors on the rate of reaction. From kinetic evidence presented (2) for a variety of other solvents, one could expect that water would markedly inhibit the reaction, and we have obtained quantitative information in support of this. On the other hand, the role of acid is not clear. Conflicting results have been published (2); p-toluene-sulfonic acid was found to increase the rate of solvolysis of clear. Conflicting results have been published (2) ; p-toluene-sulfonic acid was found to increase the rate of solvolysis of dehydroepiandrosterone sulfate in tetrahydrofuran-6% H2O, but the same acid had no effect in tetrahydrofuran-10% ethanol. Unfortunately, it is not obvious how these results can be extrapolated to a different solvent system and a different acid, viz. sulfuric acid in ethyl acetate equilibrated with water. In fact, although the previous authors (1) recognized that this technique might not be the species which undergoes cleavage. In this event, acid should increase the rate. In the absence of a detailed understanding of the mechanism and of the influence of solvent on it, predictions of the influence of acid in a particular case are speculation.

Since definitive conditions for the hydrolysis of urinary steroid sulfates are at present a prerequisite to our interpretation of much of their biochemistry, we have undertaken a study of the effect of sulfuric acid on the solvolysis of dehydroepiandrosterone sulfate in ethyl acetate. Our investigations have led us to a procedure by which the rate of reaction can be controlled through the adjustment of various defined parameters. We have also investigated the stability of 3,5-cycloandrostane-6α-ol-17-one to various times from methanol raised the melting point to 182-186° (Kofler block) (see reference (7), m.p. 192-193°). The resulting suspension was extracted with 200 ml of ethyl acetate. The organic layer was added to 2 liters of ether which had been saturated with water. The sodium salt (120 mg) precipitated on cooling to 0° for 1 hour. It formed colorless needles, m.p. 166-169°; flame photometry showed the presence of sodium and the absence of potassium. Recrystallization was dissolved in 40 ml of water and added to 60 ml of aqueous 5 NaCl. The resulting suspension was extracted with 200 ml of ethyl acetate. The organic layer was added to 2 liters of ether which had been saturated with water. The sodium salt (120 mg) precipitated on cooling to 0° for 1 hour. It formed colorless needles, m.p. 166-169°; flame photometry showed the presence of sodium and the absence of potassium. Recrystallization was carried out in part at the 44th Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois, April 11 to 15, 1960.

EXPERIMENTAL PROCEDURE

Materials—Potassium dehydroepiandrosterone sulfate was prepared according to the general procedure of Sobel and Spoerri (6). The yield of material melting at 216-218° (Kofler block), (see reference (2), m.p. 219-223°), was 40% when 2.5 times the stoichiometric amount of pyridine-SO₃ complex was used. Only a 10% yield was obtained with the recommended amount of complex.

For the preparation of the crystalline sodium salt of dehydroepiandrosterone sulfate, 200 mg of the potassium salt were dissolved in 40 ml of water and added to 60 ml of aqueous 5 NaCl. The resulting suspension was extracted with 200 ml of ethyl acetate. The organic layer was added to 2 liters of ether which had been saturated with water. The sodium salt (120 mg) precipitated on cooling to 0° for 1 hour. It formed colorless needles, m.p. 166-169°; flame photometry showed the presence of sodium and the absence of potassium. Recrystallization was carried out in part at the 44th Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois, April 11 to 15, 1960.

Ethyl acetate (reagent grade) was purified by extracting it with aqueous Na₂CO₃, then with water, drying over CaCl₂, and distilling twice. The other solvents and materials used were of a commercial reagent grade and were not further purified.Kinetic Experiments—All reactions were carried out in a thermostated (±0.3°) bath at the temperatures indicated. The disappearance of the steroid sulfate was measured by removing at appropriate times 1.0 ml of the reaction mixture, adding it to...
1.0 ml of water, removing the organic solvent under a stream of nitrogen, and analyzing the solution according to the method of Roy (8) using methylene blue. The method was modified in that the optical density of the final chloroform solution was measured directly (without dilution with ethanol) on a Beckman model DU spectrophotometer at 660 μm which we found to be the maximum of absorption of the complex produced. Beer's law was obeyed over a range of concentrations equivalent to 0 to 40 μg per ml of steroid sulfate in the aqueous phase, and all measurements were referred to a standard straight line plot of optical density against concentration. Dehydroepiandrosterone did not interfere with the color produced by the corresponding sulfate.

The kinetic experiments were performed by adding the appropriate amount of commercial reagent grade sulfuric acid (freshly dissolved in ethyl acetate) to the reaction mixture containing the sodium salt of dehydroepiandrosterone sulfate and diluting to a known volume. Solutions of the sodium salt were obtained by dissolving the crystalline material in ethyl acetate. In a few cases indicated, the solutions were prepared by directly extracting the sodium salt from a mixture of the potassium salt in aqueous 3 M NaCl.

When the logarithm of the concentration of steroid sulfate was plotted against time, a straight line was produced in all cases. The reactions were usually followed until they were 60% complete. The half-time was obtained graphically and the first order rate constant (k') was calculated from this.

In all of the experiments, unless otherwise noted, the initial concentration of steroid in ethyl acetate was 6 × 10^{-5} M. The results of the kinetic experiments are shown in Figs. 1 and 2.

Identification of Products—When pure sodium or potassium dehydroepiandrosterone sulfate was used as a starting material and sufficient time (>5 times the half-time period) was allowed for the reaction to proceed essentially to completion, dehydroepiandrosterone was identified as the product in the following way. The ethyl acetate solution was washed with aqueous 50% sodium carbonate solution, then with water, and finally dried over anhydrous sodium sulfate. The residue left after removal of the solvent at reduced pressure was chromatographed on paper in a ligroin-propylene glycol system of solvents (9). The rate of movement of the product was identical with that of a standard sample of dehydroepiandrosterone. On a strip of paper 45 cm long, no other 17-ketosteroid (determined by the Zimmermann color) was observed after a development time which allowed the dehydroepiandrosterone to move 12 cm. The yield of dehydroepiandrosterone, as measured by the intensity of the Zimmermann color before chromatography, was essentially quantitative. The product of reaction was identified in this way as dehydroepiandrosterone from runs made with a variety of concentrations of water and sulfuric acid.

Analysis of Urine—A 24-hour specimen of urine (pH 6.1) from a 20-year-old white male was neutralized with aqueous 5% Na₂CO₃ solution. One-third of the final sample was made 3 M with sodium chloride and extracted once with two volumes of ethyl acetate. The organic phase was dried with 6 g of anhydrous sodium sulfate (commercial analytical reagent) for every 100 ml of solution. To the dried and filtered extract was added sufficient sulfuric acid in ethyl acetate to make the final reaction mixture 2.0 × 10^{-4} M in sulfuric acid. The solution was then kept at 30° for 16 to 20 hours, extracted with 100 ml of aqueous 1 N NaOH solution and twice with 100 ml of water. The washed solution was dried over sodium sulfate and evaporated to dryness under reduced pressure at a temperature not exceeding 40°. The residue (15 mg) was transferred with several portions of benzene (total volume, 4.0 ml) to a column (9 mm diameter) of 6.0 g of alumina (Woelm, neutral) which had been desactivated with 4% of water (14). The column was eluted with a mixture of solvents which was continuously graded (14) by a siphon system from 0.2% ethanol in benzene (140 ml) toward 2.0% ethanol in benzene. Fractions of 0.9 ml were collected and an aliquot of each was analyzed quantitatively by the Zimmermann method. Another aliquot of the fractions was submitted to further chromatography on paper using a ligroin-propylene glycol system of solvents. Dehydroepiandrosterone, identified by paper chromatography, was eluted from the column with a peak fraction at Fraction 40. A standard sample was eluted in the same fractions in another chromatogram. The total weight of material from...
the urine aliquot in the fractions of this elution band was 730 μg, equivalent to 2.3 mg for the whole specimen. Recovery experiments showed we obtained a 65 to 75% yield. The corrected excretion was, therefore, 3.3 mg/24 hours for the person examined.

No measurable amount of ethylocholanolone was observed in fractions near Fraction 60 where a standard sample was found to be eluted. A standard sample of androsterone was eluted near Fraction 53, and at this point in the chromatogram, the urine specimen showed the presence of less than 0.2 mg/24 hours.

The steroid was eluted from the column with a peak fraction corresponding fractions from the urine specimen showed that this compound (3,5-cycloandrostan-6β-ol-17-one) was not present.

Experiments with 3,β-cycloandrostan-6β-ol-17-one—the steroid was prepared from dehydroepiandrosterone tosylate by a modification of the procedure previously reported for polyunsaturated steroids (10). The main difference in the method was that the time of reaction was extended to 3 hours in view of the lesser ease with which monounsaturated (Δ4) steroids undergo the 3,5-cyclosteroid rearrangement. The yield of material, recrystallized several times from acetone and melting at 138-140°, was 46% (see reference (11), m.p. 136-138°).

When the i-steroid was submitted to the hydrolytic conditions in ethyl acetate (2 × 10⁻⁴ M sulfuric acid, 30°, 18 hours) and the product was separated and identified by chromatography on alumina and then on paper as in the procedures described above, only starting material (90%) was recovered. The same result was obtained when the i-steroid (500 μg) was added to neutral urine (1/4 of a 24-hour specimen) followed by extraction, etc. The i-steroid was eluted from the column with a peak fraction near Fraction 38.

RESULTS AND DISCUSSION

Extraction of the potassium salt of dehydroepiandrosterone from a neutral aqueous solution which was 3 M in NaCl with ethyl acetate brought the sodium salt of the steroid into the organic phase. Its presence in the ethyl acetate was firmly established by the addition of ether which caused it to precipitate in a crystalline form. The steroid salt proved to be quite stable in the organic solvent; less than 1% reaction took place at 30° during 5 days, and only upon the addition of sulfuric acid was there a significant conversion of the ester to the free alcohol. The reaction was first order in steroid and, over a definite range of concentrations, first order in sulfuric acid; as the concentration of added acid was increased above 10⁻⁴ M, however, the order in sulfuric acid approached zero (Fig. 1). Nevertheless, sulfuric acid catalyzed the reaction at concentrations considerably in excess of the stoichiometric amount of steroid salt. We believe these findings clearly demonstrate that added acid catalyzed the over-all hydrolysis of the sulfate ester in ethyl acetate.

As proposed earlier (2), the over-all "hydrolysis" appears to be a solvolysis, and water itself, if anything, detrimental. This is demonstrated clearly in Fig. 2 in which a sharp decrease in the rate constant is seen to occur as the water content of the medium is increased. A decrease in the energy of activation when going from 6 to 3% water in tetrahydrofuran has already been reported (2), and it would appear that this is a general effect of water. We have, therefore, an interesting example of a compound, dehydroepiandrosterone sulfate, which can undergo solvolysis in two very different ways: (a) with S-O cleavage yielding dehydroepiandrosterone, and (b) with R-O cleavage yielding the i-steroid (5), depending on the conditions of the reaction.

To use solvolysis in ethyl acetate as an exact procedure for the hydrolysis of steroid sulfates, we have quantitatively studied the factors which have an important influence on the rate constant. They are (a) the acidity (Fig. 1), (b) the concentration of water (Fig. 2), and (c) the temperature at which the reaction is carried out. The rate constant varies from 0.539 hours⁻¹ at 30° to 0.19 hours⁻¹ at 20° at a sulfuric acid concentration of 2.0 × 10⁻⁴ M in ethyl acetate containing 1.0% water by volume. This corresponds to a 2.7-fold increase in the half-time period for the 10° decrease in temperature indicated. An even greater temperature dependence would be expected at lower concentrations of acid, and earlier data (2) suggest that this is so. It is evident from Figs. 1 and 2 that a factor of 2 powers of 10 can be introduced into the half-time period by varying the concentration of sulfuric acid from 10⁻⁴ to 10⁻² M and that a factor of 160 can be produced by varying the concentration of water from 0% to the maximal solubility of 3.1% at 30°. A fourth and subtle effect is derived from the influence of water. In the analysis of natural, aqueous solutions, e.g., urine, the sulfates(s) are extracted with the ethyl acetate. The curves in Fig. 3 show that the solubility of water in ethyl acetate is significantly dependent on temperature. In particular, we have found that, when ethyl acetate is partitioned against aqueous 3 M NaCl, the content of water in the organic phase rises from 1.8% at 15° to 2.7% at 35°. Analogous but slightly higher percentages have previously been determined (12) for pure water and they were verified by us to within 0.2% water content. Our measurements were made using the intensity of the absorption band for water in the infrared region near 1.92 μ. When the values for the water content are compared with the data in Fig. 2 for the effect on the rate constant, it is evident that reasonable variations in room temperatures at which the extractions are carried out can have a 3-fold effect on the half-time period.

From these observations, it is clear that to approach a 100% yield in the hydrolysis, which is essential in the quantitative analysis of urine, a carefully controlled sequence of steps must be carried out.

A simple procedure for the extraction, solvolysis, and analysis of urinary 17-ketosteroids is described in the experimental section; 3,β-cycloandrostan-6β-ol-17-one was stable to the conditions both of reaction and separation. Since 6-substituted 3,5-cyclosteroids can react as much as 10⁶ times as fast as the corresponding 3-substituted Δ4-stereoids in solvolytic reactions (13), we consider the stability of the i-steroid to our procedure.

![Fig. 3. Equilibrium concentration of water in ethyl acetate at various temperatures after the organic solvent is shaken with pure H₂O (-- --) (recalculated from the weight-per-cent data given in reference (12)) and with 3 M aqueous NaCl (---).](http://www.jbc.org/)

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as evidence that unesterified steroidal 3-alcohols present in the urine do not undergo alkyl-oxygen cleavage during the solvolysis. Furthermore, since the sulfates would be expected (2) to undergo S-O cleavage, our procedure should liberate alcohols from sulfate esters without artifact formation, and indeed dehydroepiandrosterone was identified from a white male, 20 years old, at an excretion rate of 3.3 mg/24 hours. No 9-steroid was found. Of further interest, no etiocholanolone and only a trace amount, if any, of androsterone was observed. Since it is well known (e.g., reference (14)) that these latter metabolites are present in human urine as the glucosiduronates in amounts equal to or greater than that of dehydroepiandrosterone, the method we are describing seems to have the property of differentiating the free steroids or sulfate esters from the glucosiduronates. In all normal urines which we have examined, etiocholanolone has been absent in the final products or present in amounts on the borderline of detection (ca. 0.1 mg/24 hours). The "two-phase" method reported in the literature (1) which utilizes an acidified (H₂SO₄) urine and solvolysis in diethyl ether has also been found (1) to yield only very small amounts of androsterone relative to dehydroepiandrosterone. Quite recently, Burstein et al. (15) have shown that sulfuric acid is not a good catalyst for the hydrolysis of glycosides in organic solvents containing water which might account for the selectivity of the procedure, but it might also arise from different partition coefficients of the sulfates and glycosides between water of high ionic strength and ethyl acetate. This point is being investigated.

SUMMARY

1. The solvolysis of sodium dehydroepiandrosterone sulfate in ethyl acetate has been found to be catalyzed by sulfuric acid, and quantitative data on the additional effect of water and temperature are reported; based on these findings, a procedure for the examination of urine is described.

2. The extraction of dehydroepiandrosterone sulfate from a neutral urine, its solvolysis in ethyl acetate, and its separation and quantitative determination are described.

3. 3,5-Cycloandrostan-6β-ol-17-one was stable to the entire isolation procedure and was found to be absent in some normal urine specimens.

4. Etiocholanolone was not observed in the final products from the examination of urine indicating the method described differentiates the free steroids and their sulfate esters from the glucosiduronates.

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REFERENCES

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