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Corticosteroid 21-Sulphates in Human Urine

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Pasqualini (1958, 1960) studied the distribution of the corticosteroids between the free, sulphate and glucuronide fractions in the urine of subjects after administration of adrenocorticotrophic hormone and established that an important fraction of corticosteroids was excreted in the urine as conjugates, contrary to the usual opinion (Neher, 1958; Peterson, Wyngaarden, Guerra, Brodie & Bunin, 1955; Peterson & Pierce, 1960). Corticosterone and 11-dehydrocorticosterone are found mainly in the ester sulphate fraction. The aim of the present research was to demonstrate the existence of corticosterone 21-sulphate and cortisol 21-sulphate in human urine.

EXPERIMENTAL AND RESULTS

Paper chromatography. Table 1 gives the *R* values of synthetic corticosteroid 21-sulphates relative to that of deoxycorticosterone 21-sulphate (Pasqualini, Zelnik & Jayle, 1960). For hydrolysis of the conjugates the developed dried chromatogram was suspended at 40° for 3.5 hr. in a covered cylindrical glass jar containing a mixture of conc. hydrochloric acid and dioxan (1:3, v/v) (Schneider & Lewbart, 1956).

Identification of corticosterone sulphate

Corticosterone was administered orally to a normal subject in three 100 mg. doses at 8 hr. intervals. The urine was collected during the 48 hr. after the first dose. The

Table 1. *R* values of corticosteroid 21-sulphates

The solvents used were: system 1, toluene-*n*-butyl acetate-butanol-methanol-4*N*-NH₃ soln.-water (3:6:1:5:5, by vol.); system 2, toluene-*n*-butyl acetate-butanol-methanol-acetic acid-water (8:10:2:10:1:9, by vol.); system 3, propan-2-ol-hexane-NH₃ soln. (sp.gr. 0.925)-water (40:10:28:20, by vol.) (Cavina & Tentori, 1958). Whatman no. 1 filter paper and the descending technique were used. System 1 was run for 20 hr., system 2 for 40 hr. and system 3 for 16 hr. Temperature was $24 \pm 1^\circ$.

Compound	<i>R</i> relative to deoxycorticosterone 21-sulphate in system		
	1	2	3
Pyridinium deoxycorticosterone sulphate	1	1	1
Sodium deoxycorticosterone sulphate	1	1	1
Pyridinium corticosterone sulphate	0.40	0.58	0.70
Pyridinium cortisol sulphate	0.25	0.35	0.58

Table 2. *Urinary steroid output*

Steroid administered and determined	Steroid excreted ($\mu\text{g./l.}$)		
	Free	Ester sulphate	Glucuronide
Corticosterone	5	800	100
Cortisol	1200	700	120

urine, to which solid ammonium sulphate was added to give a 50% (w/v) solution, was extracted three times at pH 7 with an equal volume of ether-ethanol (3:1, v/v) (Edwards, Kellie & Wade, 1953). The extractions were made in separating funnels with continual shaking during 1 hr. The ethereal extracts were combined and dried. The dry urine extract was dissolved in water and the free corticosteroids were extracted by dichloromethane. The aqueous solution containing the conjugated corticosteroids was evaporated to dryness. These corticosteroids were separated on alumina into the sulphate and glucuronide fractions as described by Crepy, Jayle & Meslin (1957).

The ester sulphate fraction was purified by rechromatographing on alumina and, after elution, the ester sulphate fraction was further purified by dissolution in acetone-chloroform (3:1, v/v). This eliminated materials insoluble in this solvent mixture. The ester sulphate fraction of urine extracts was chromatographed first in solvent mixture 3 (Table 1). The zone that corresponded to the synthetic corticosterone sulphate used as a marker was eluted, and portions were chromatographed in solvent mixtures 1 and 2 (Table 1). The zone in solvent mixture 2 corresponding to the synthetic corticosterone 21-sulphate was eluted and hydrolysed by purified sulphatase obtained from *Helix pomatia* (Jarrige & Lafoscade, 1959). The liberated steroid was chromatographed with chloroform or benzene on paper treated with formamide, with toluene on paper treated with propanediol and, after acetylation, with cyclohexane-benzene (1:1, v/v) on propanediol-treated paper (Burton, Zaffaroni & Keutman, 1951; Zaffaroni, 1953).

With the three solvents of Table 1 the zones on the chromatograms corresponding to corticosterone 21-sulphate gave positive methylene-blue reactions (Vlitos, 1953; Crepy & Judas, 1960); this is a colour reaction for ester sulphate. In the chromatogram with solvent mixture 1, the ester sulphate was identified by the rhodizonate test (Burma, 1953) and by the blue tetrazolium reaction, which was positive after hydrolysis in dioxan-hydrochloric acid mixture. Eluates from the zones showed λ_{max} 254 m μ . The free steroid obtained after hydrolysis with sulphatase had

the same R_F as corticosterone in the three solvent systems and had λ_{max} 254 m μ . It gave a positive blue tetrazolium reaction and a yellow fluorescence in ultraviolet light. Its acetate behaved chromatographically as authentic corticosterone acetate.

Identification of cortisol sulphate

Cortisol was administered orally to a normal subject in three 100 mg. doses at 8 hr. intervals. The urine was collected during the 36 hr. after the first dose. The urine, to which 5% (w/v) of sodium chloride had been added, was extracted three times with an equal volume of butanol at pH 7. The dried extract was dissolved in water and the free corticosteroids were extracted by dichloromethane. The conjugated corticosteroids were separated on alumina into sulphate and glucuronide fractions. The purification of ester sulphate fraction was the same as that used for corticosterone sulphate. The purified corticosteroid sulphate was hydrolysed with dioxan to which 3% (w/v) of trichloroacetic acid had been added (Cohen & Oneson, 1953).

Paper electrophoresis. Ethanol containing the ester sulphates was placed on strips of Carl Schleicher and Schüll, Dassel, no. 2043 (Zurich, Riedlistra 15, Switzerland) paper, in an apparatus of the Grassmann & Hannig (1952) type. The electrophoresis was at 60 v for 7 hr. at 1 ma/cm. at 22° with 0.3*N*-ammonium carbonate buffer, pH 8.8.

Paper chromatography. The ester sulphates were successively fractionated in the three solvent systems of Table 1. A fraction of the eluate from the zone corresponding to the synthetic cortisol 21-sulphate, which had been chromatographed with solvent 1, was hydrolysed by sulphatase. Another part was hydrolysed with dioxan-trichloroacetic acid solution (Cohen & Oneson, 1953). The liberated steroid was chromatographed with chloroform on formamide-treated paper and in toluene-ethyl acetate-methanol-water (9:1:5:5, by vol.) (Bush, 1952), and its acetate with toluene on propanediol-treated paper. The free steroid and the acetate had the same migration characteristics as cortisol and cortisol acetate respectively. A further part of the extract was subjected to electrophoresis with synthetic cortisol sulphate as marker. The material was eluted and hydrolysed, and the liberated steroid was chromatographed with chloroform on formamide-treated paper. It had the same R_F as cortisol.

Table 2 gives the relative distribution of corticosterone and cortisol in the free, ester sulphate and glucuronide fractions.

SUMMARY

1. After administration of 300 mg. of corticosterone or cortisol to a man, the greater part of these two corticosteroids were found in the urine in ester sulphate fraction.

2. Evidence that sulphate was attached to C-21 was provided by the similarity of chromatographic behaviour with that of authentic corticosterone 21-sulphate or cortisol 21-sulphate, electrophoretic separation for cortisol sulphate and the reaction to tetrazolium being positive only after hydrolysis.

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The Biosynthesis and Metabolism of Polyols

SORBITOL (D-GLUCITOL) OF PLUM LEAVES

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Acyclic polyhydric alcohols occur in many species of plants (Barker, 1955), although in the majority of cases neither quantitative data on the amounts present nor information on their metabolic role is available. Hypotheses have been advanced to explain the metabolism of polyols with little supporting evidence. Thus Donen (1939) suggested that sorbitol is formed in plum fruit when the concentration of hexoses reaches a critical level, whereas Kidd, West, Griffiths & Potter (1938) found that much of the sorbitol in pears is replaced by hexulose (probably fructose) as the fruit ripens. Sorbitol can also be utilized for polysaccharide synthesis in the leaves of Rosaceae and Gramineae (Barker, 1955; Altermatt & Neish, 1956), but nothing is known of the extent of this conversion under natural conditions.

Experiments with $^{14}\text{CO}_2$ originally designed to yield information on the biosynthesis of leaf poly-

saccharides (Andrews & Hough, 1958) indicated that sorbitol, the acyclic polyol in plum leaves, has an important role in the metabolism of these leaves. The incorporation of ^{14}C into sorbitol was considerably greater than into starch, and this observation initiated further studies of sorbitol metabolism in plum leaves.

Adcock (1957) assayed sorbitol by oxidation with sodium periodate and subsequent estimation of the two molecular equivalents of formaldehyde produced from the primary alcohol groups (O'Dea & Gibbons, 1953). Before oxidation, interfering substances were degraded by heating in sodium carbonate solution, and the degradation products removed by treatment with ion-exchange resins. However, when applied to plum-leaf extracts Adcock's method gave inconsistent results, a conclusion supported by White (1958), who suggested a preliminary acid hydrolysis of non-reducing carbohydrates such as sucrose to facilitate their degradation and removal. A simpler method

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