Formation of Hydrogen Sulphates as a Means of Separating Alcoholic from Non-alcoholic Steroids

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Sobel, Drekter & Natelson (1936) determined plasma cholesterol by a method that involved the formation of cholesteryl hydrogen sulphate as a means of separating the sterol from its esters and from other non-alcoholic substances. It is now shown that under appropriate conditions the analytical approach of Sobel *et al.* (1936) is capable of wider application.

Each compound tested (see Table 1) was dissolved in dry pyridine (0.2 ml), and the solution was chilled in ice-water and mixed with a saturated solution of chlorosulphonic acid in carbon tetrachloride (0.1 ml; a fresh solution was prepared weekly). After 30 min at room temperature, 0.4 M-NaHCO₃ (1.0 ml) was added and the non-alcoholic fraction extracted with benzene (2 × 2.5 ml) or (in the cases of cortisol and aldosterone) with dichloromethane (2 × 2.5 ml). The extract was washed with water (0.5 ml) and evaporated to dryness. For solvolysis the aqueous phases were combined and diluted with 2 M-pyridinium sulphate (1.0 ml) and extracted with dichloromethane (2 × 2.5 ml), and the extract was evaporated to dryness; the residue was kept in dioxan (1 ml) for 4 h at 37°C, and the solution was diluted with dichloromethane (4 ml), washed with 0.4 M-NaHCO₃ (0.5 ml) and evaporated to dryness (McKenna & Norzymski, 1960), giving the alcoholic fraction. In the case of pregnanediol the aqueous phase was adjusted to pH 1, mixed with ammonium sulphate (0.75 g) and extracted with ether-ethanol (3:1, v/v; 3 × 2 ml) (Edwards, Kellie & Wade, 1953); solvolysis was in dioxan containing 2% (v/v) of ethanol at 47°C. For enzymic hydrolysis the aqueous phase was adjusted to pH 4.9 with acetic acid; 2 M-sodium acetate buffer, pH 4.9 (0.2 ml), was added and then the gastric juice of *Helix pomatia* (Henry & Thevenet, 1952; supplied by l'Industrie Biologique Française, Gennevilliers, France) containing 8000 sulphatase units (Roy, 1956). After 48 h at 37°C the mixture was extracted with dichloromethane (3 × 2 ml), and the extract was washed with 0.4 M-NaHCO₃ (0.5 ml) and evaporated to dryness. The minor fraction and part of the major fraction (see Table 1)

were taken for counting of radioactivity. The remainder of the major fraction was chromatographed on paper (for systems see Table 1) and the chromatogram scanned for radioactivity.

Each compound tested was recovered in the appropriate fraction, unchanged (chromatographic evidence) and in high yield (see Table 1). Two consecutive fractionations of [1,2-³H]4-androstene-3,17-dione gave 0.3% of radioactive material in the first and 0.2% in the second alcoholic fraction, whereas in the case of 11 β -hydroxy[1,2-³H]-4-androstene-3,17-dione the corresponding values were 1.5 and 0.5% respectively. This suggests that in the first case the presence of radioactivity in the 'wrong' fraction was due largely to a factor (or factors) inherent in the method and in the second case to an impurity.

The recovery of parent alcohols from their respective sulphates was brought about either by solvolysis with dioxan or by enzymic hydrolysis, but not in every instance were both methods effective. Thus solvolysis of compounds (8), (9) and (10) (see Table 1) gave rise to unidentified by-products and enzymic hydrolysis of compounds (6) and (7) was incomplete (15-25%). Moreover in the case of pregnanediol the conditions of extraction of the sulphate fraction and of its solvolysis had to be modified. Consequently experimental conditions should be chosen to suit each particular case. The usefulness of the fractionation procedure is illustrated by its application to the determination of progesterone and related substances suggested some time ago (Norzymski, 1966) and based on the argument that an alcoholic fraction obtained from the reduction of a non-alcoholic fraction with 20 β -hydroxy steroid oxidoreductase should contain only 20 β -hydroxypregnanes derived from corresponding 20-oxopregnanes lacking reactive hydroxyl groups.

Pooled urine (4.0 litres) collected during the last month of human pregnancy was mixed with [1,2-³H]progesterone (6.0 ng; 1.2 × 10⁶ d.p.m.) and extracted with light petroleum (b.p. 60-80°C; 2 × 1 vol.), and the extract was washed with M-NaOH (2 × 0.05 vol.) and then with water. A portion (0.64) of the extract was fractionated as outlined above but with tenfold amounts of solvents and

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Table 1. Recoveries of some steroids in alcoholic and non-alcoholic fractions

The values given for the percentage recoveries in the alcoholic and non-alcoholic fractions are the means of three determinations. The solvent systems used were: I, 2,2,4-trimethylpentane-methanol-water (10:10:1, by vol.); II, cyclohexane-benzene-methanol-water (5:2:5:1, by vol.); III, cyclohexane-nitromethane-methanol (2:1:1, by vol.); IV, cyclohexane-benzene-methanol-water (2:2:2:1, by vol.); V, benzene-methanol-water (4:2:1, by vol.).

Compound	Quantity		% found		System in which major fraction chromatographed
	(μg)	(d.p.m.)	In alcoholic fraction	In non-alcoholic fraction	
(1) [1,2- ^3H]4-Androstene-3,17-dione	20	108 000	0.3	97	I
	0.001	220 000	1.1	94	I
(2) 11 β -Hydroxy-[1,2- ^3H]4-androstene-3,17-dione	0.4	22 000	1.4	93	II
(3) [1,2- ^3H]Progesterone	5	65 000	0.3	96	I
(4) [6,7- ^3H]Oestrone methyl ether	10	50 000	0.5	96	III
(5) 20 β -Hydroxy-[1,2- ^3H]4-pregnen-3-one	8	14 000	94	0.6	I
(6) [1,2- ^3H]5 β -Pregnane-3 α ,20 α -diol	1	65 000	93	0.3	II
(7) [1,2- ^3H]Testosterone	20	105 000	97	0.4	II
(8) [4- ^{14}C]Corticosterone*	5	19 000	93	1.5	IV
(9) [4- ^{14}C]Cortisol*	10	18 000	90	1.1	V
(10) [1,2- ^3H]Aldosterone*	5	70 000	93	2.1	V

* The sulphate fraction was treated with the juice of *Helix pomatia*, in all other instances with dioxan.

reagents. One-tenth of the non-alcoholic fraction was reduced with 20 β -hydroxy steroid oxidoreductase (supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) essentially as reported by Hübener & Sahrholz (1959). The alcoholic fraction was separated as above (solvolysis with dioxan) and partitioned between aqueous ethanol (1.0ml; 70%, v/v) and light petroleum (2 $\times$ 1.0ml). The radioactivity in the former phase accounted for 41% of the added [^3H]progesterone. G.l.c. of a sample (corresponding to 31ml of urine) on 1% SE-30/Gas-Chrom Q at 210°C gave, apart from some minor components found also in the water blank, only three components with the retention times of 20 β -hydroxy-5 β -pregnan-3-one, 20 β -hydroxy-5 α -pregnan-3-one and 20 β -hydroxy-4-pregnen-3-one (peak height proportions 7:1:4).

The nature of the operations preceding chromatography gives confidence in the assignment of structure on the basis of chromatographic behaviour and requires that the three compounds detected derive respectively from urinary 5 β -pregnane-3,20-dione, 5 α -pregnane-3,20-dione and progesterone. Confirmation of identity was obtained by g.l.c. after acetylation and after reduction with borohydride followed by acetylation. In the latter case the characteristic formation of two products with the retention times of isomeric 20 β -acetoxy-pregnadienes constitutes very strong additional evidence for the presence of 20 β -hydroxy-4-pregnen-3-one in the chromatographed sample (Norymberski & Riondel, 1970) and hence of progesterone in the urine. The concentration of

progesterone in the analysed pool was determined to be approx. 4 $\mu\text{g/l}$, in reasonable agreement with values determined by much more laborious methods (Ismail & Harkness, 1967; van der Molen & Corpéchet, 1968; Schubert, Wehrberger, Wachtel & Schlegel, 1969). Although the present results clearly establish the practicability of the employed analytical approach, any claim to the intrinsic specificity of the method must be qualified by the finding of 3 α -hydroxy steroid oxidoreductase activity in preparations of the enzyme used (Pocklington & Jeffery, 1967).

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