INVESTIGATION INTO THE SULPHOCONJUGATION OF 5α-ANDROST-16-EN-3β-OL BY PORCINE LIVER

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1. Introduction

The biosynthesis of 16-androstene steroids by boar testis and the possible physiological role of two of them has been well documented [1,2]. The end products of the biosynthetic pathway are the epimeric 3-alcohols, an-α and an-β. An-α may be pheromonally active in pigs, but the boar testis produces greater quantities of the β-alcohol, which at present has no known physiological action, as the free steroid [1]. However, little is known about the sulphate and glucuronide conjugates of these steroids, except that the boar is capable of producing them from pregnenolone and that testicular tissue appears to produce more an-β sulphate than an-β [3]. The mechanism of converting free steroid alcohols to their corresponding sulphate esters has been elucidated and is now considered to be a three stage process:

(i) The synthesis of adenosine-5′-phosphosulphate (APS) from ATP and sulphate ions;
(ii) The formation of phosphoadenosine-5′-phosphosulphate (PAPS) from APS and ATP;
(iii) The transfer of the sulphate from PAPS to the steroid [4].

Steroid sulphates can be metabolised in the same way as the free steroids, for instance 17-hydroxypregnenolone-3-sulphate can be converted to DHA-3 sulphate [5] and more recently [6] pregnenolone sulphate has been found to be converted to andien-β sulphate by boar testis tissue. The physiological role of steroid sulphates is not yet fully understood. In plasma, they may be a circulating store of androgen and oestrogen precursors, due to their greater polarity and solubility compared to the free steroids.

Here we describe the synthesis and properties of an-β sulphate, found to be unstable in the crystalline form [3] and also the results of investigations into the sulphokinase of porcine liver.

Steroid abbreviations and trivial names: an-β, 5α-androst-16-en-3β-ol; an-α, 5α-androst-16-en-3α-ol; 17-hydroxypregnenolone, 3β, 17α-dihydroxy-5-pregnen-20-one; DHA, 3β-hydroxy-5-androsten-17-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one; andien-β, 5,16-androstadien-3β-ol; 5α-androstenone, 5α-androst-16-en-3-one; androsterone, 3α-hydroxy-5α-androstan-17-one

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2. Materials and methods

Authentic steroids, cofactors, scintillators, solvents and materials for thin-layer chromatography (TLC) were as in [7]. [5α,6α-^{3}H]5α-Androstenone (21 Ci/mmol) was purchased from ROTOP, Isocommerz GmbH, Dresden. Alumina for chromatography (Peter Spence Ltd, Widnes, Lancs.) was baked to constant weight at 150°C and shaken for 6 h with distilled water (5.5%, v/w).

2.1. *Preparation of an-β sulphate pyridinium salt*

An-β (50 mg) was dissolved in freshly distilled pyridine (5 ml), and chlorosulphonic acid (0.1 ml) added slowly. The mixture was allowed to cool and shaken to redissolve any precipitate. Light petroleum (b.p. 40–60°C) (2 ml) was introduced, whereupon extensive precipitation occurred. When precipitation was complete the liquid was removed and centrifuged (bench centrifuge). The clear liquid was decanted into a clean vessel and light petroleum (b.p. 40–60°C) (28 ml) added. The flask was left overnight at 4°C to allow the an-β sulphate crystals to settle. The liquid was removed and the solid recrystallised from hot

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methanol (2 X) whereupon colourless crystals were obtained (yield 30%). These crystals were examined by TLC in two systems. When run in ethanol:benzene: butanone:water (3:3:3:1, by vol.) a single spot was detected using I_2 vapour and Allen reagent [8]. The R_F value of this spot was 0.71 whereas that of an-β was 0.89. When run in benzene:methanol (9:1, v/v), the crystals again provided a single spot with an R_F value of 0.10, compared to that of 0.73 for an-β in this system. When sprayed with Allen reagent and heated to 110°C, this spot gave an identical colour to an-β, brick red turning blue on cooling. Infrared spectroscopy (dry KBr disc) (Perkin Elmer 157G Grating Infrared Spectrophotometer) showed the following absorption bands; 3030 (C–H stretch of olefin); 1614 (C = C stretch); 1200–1260 (characteristic of 3-sulphate) [9] and 715 cm^{-1} (C–H bend of 16-ene). On heating, the compound decomposed irreversibly at 122°C, the melting point of an-β. TLC (benzene:diethyl ether 9:1, v/v, run twice) of the melted an-β sulphate, exhibited two spots; one was found to be an-β, but the bulk of the material migrated as a single spot (16 cm above origin). Neither an-β itself nor any of the 16-androstenes decomposed at their melting points.

Mass spectrometry (LKB 2091 Mass Spectrometer) of an-β sulphate at 110°C (fig.1) showed that the compound decomposed, because no peaks at *m/e* 433 (the expected molecular ion) were evident. The peaks at *m/e* values of 274, 258 and 256 may correspond to free an-β, 5α-androst-16-ene and a diene with one double bond at C-16 and another in ring A. A rise to 150 and 200°C caused the peak height at *m/e* 274 to diminish while those at *m/e* 258 and 256 increased. Thermal decomposition of DHA sulphate and androsterone sulphate has been shown to give rise to such unsaturated derivatives [10]. The heat instability of an-β sulphate could thus explain the finding that its melting point and that of the parent steroid alcohol are identical. At room temperature an-β sulphate was stable in the crystalline form for ⩾3 months when stored in a dessicator, and in methanol at 4°C for ⩾1 month. The stability of this preparation may be due to the precipitation of polar acid salts by the first aliquot of light petroleum (b.p. 40–60°C).

Solvolysis with tetrahydrofuran [11] yielded an-β only as determined by TLC and this was verified by gas–liquid chromatography (GLC). This was performed on a Series 104, Model 24, dual flame ionization chromatograph (Pye-Unicam, Cambridge). An-β was quantified on a silanized glass column containing diatomite CLQ (100–120 mesh) coated with CDMS (0.6%, v/w) plus OV-101 (0.75%, v/w). Carrier gas (argon) flowrate was 50 ml/min and the oven was maintained at 219°C. The relative retention time of an-β (5α-cholestane = 1.00) was 0.27 (6.8 min).

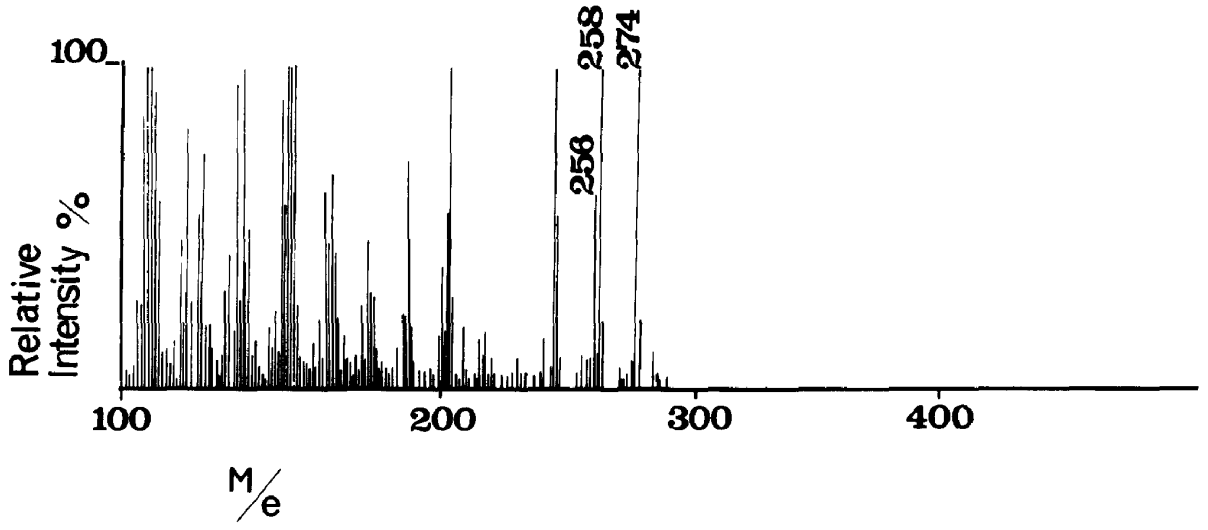

Fig.1. Mass spectrometry (LKB 2091 Mass Spectrometer) of an-β sulphate was performed at 110°C. No peaks were evident at *m/e* 433 indicating the total breakdown of steroid conjugation. The peak at *m/e* 274 may be free an-β while the peaks at *m/e* 258 and 256 may be 5α-androst-16-ene, and a diene of this in which unsaturation of ring A may have occurred.

2.2. *Assay of an-β sulphokinase*

Porcine liver was obtained from the abbatoir (Farmeat, Ashford, Kent) and transported in ice to the laboratory and used the same day. Subcellular fractions were obtained by differential centrifugation on a 50% homogenate in: Tris, 50 mmol/l; EDTA, 1 mmol/l; KCl, 150 mmol/l; pH 7.4. Protein concentrations were assessed by the method in [12].

[5α,6α-^{3}H]An-β was prepared by reduction of [5α,6α-^{3}H]5α-androstenone with KBH_4, and purified on alumina column chromatography [1]. Incubation tubes contained 4×10^5 dpm tritiated substrate and 1 ml buffered ATP (KH_2PO_4, 100 mmol/l; K_2SO_4, 10 mmol/l; $MgCl_2$, 25 mmol/l and ATP, 2 mmol/l) pH 7.4. The reaction was started by the addition of 1 ml tissue fraction (pH 7.4) and terminated at the appropriate time (usually 1 h) by the addition of diethyl ether (1 ml), and 100 μg an-β sulphate added as carrier. The aqueous phase was extracted with diethyl ether (4 × 1 ml) to remove unreacted substrate. The aqueous phase was then extracted with butan-1-ol (3 × 1 ml) to extract steroid sulphates. A selection of these extracts was assessed for an-β and an-β sulphate content and it was found that no free [5α,6α-^{3}H]an-β contaminated the butan-1-ol fractions. The butan-1-ol was removed by reduced pressure (37°C) and the residue treated with tetrahydrofuran (5 ml), overnight, to solvolyse the steroid sulphates. The tetrahydrofuran was removed by reduced pressure and the residue prepared for quantification of the an-β, by GLC and scintillation counting as in [13]. This procedure was used to investigate: (i) The subcellular distribution of the sulphokinase system; (ii) the pH dependence; (iii) the influence of ATP concentration; (iv) the rate of an-β sulphate formation; and (v) the solubilization of the enzyme system from the microsomal membranes.

3. Results and discussion

3.1. *The subcellular distribution of an-β sulphokinase system*

The enzyme system was found in both the microsomal fraction (spec. act. 0.68 pmol.mg protein^{-1}. min^{-1}) and the cytosol (spec. act. 0.54 pmol.mg protein^{-1}. min^{-1}), basal levels being found in the 1000 × *g* and 10 000 × *g* pellets (table 1). The basal level observed in the postmitochondrial supernatant cannot yet be explained, but is of considerable interest. Sulphokinases have been found in the cytosol of rat and rabbit liver and human adrenal tissue [14–16] but not previously associated with the microsomal fraction. However, rat liver may contain several sulphokinases of different substrate specificities rather than one system with a varying preference of substrate [17]. It is possible that such systems are associated with microsomal membranes as well as the cytosol. Here ATP has been used in the assay and we can therefore infer that the other enzymes in the system, namely, ATP sulphurylase, APS kinase and steroid sulphotransferase, are present in both the microsomal and cytosolic fractions of porcine liver.

Table 1
Subcellular distribution of an-β sulphokinase

Tissue fraction	An-β sulphate formed (pmol.mg protein^{-1}. min^{-1})
Homogenate	0.3
Pellet 1000 × *g*	0.07
Pellet 10 000 × *g*	0.24
Supernatant 10 000 × *g*	0.22
Pellet 176 000 × *g*	0.72
Supernatant 176 000 × *g*	0.53
Boiled homogenate	0.14

Subcellular fractions were obtained by differential centrifugation of pig liver homogenate and assayed for sulphokinase activity by incubating with [5α,6α-^{3}H]an-β. The an-β sulphate formed was quantified as in section 2

3.2. *Optimum pH and the influence of ATP concentration on the microsomal sulphokinase*

Fig.2 shows that the microsomal sulphokinase has an optimum pH 7.4 and therefore this pH was used for all subsequent investigations. The sulphotransferase of the human adrenal gland also has an optimum pH 7.4 [16] but the optimum pH of PAPS synthesis is 8.0.

The concentration of ATP in the reaction mixture appeared not to influence the rate of an-β-sulphate production up to 4 mmol/l as shown by the following results: 0.14, 0.14 and 0.15 pmol.mg protein^{-1}. min^{-1} in the presence of 0, 2 and 4 mmol ATP/l, respectively. The high yield of an-β sulphate in the incubation without added ATP may indicate that an endogenous supply of ATP was available to the enzyme. Obviously, the activity of the sulphokinase varies from preparation to preparation because the yields of an-β sulphate in this experiment were all found to be lower than

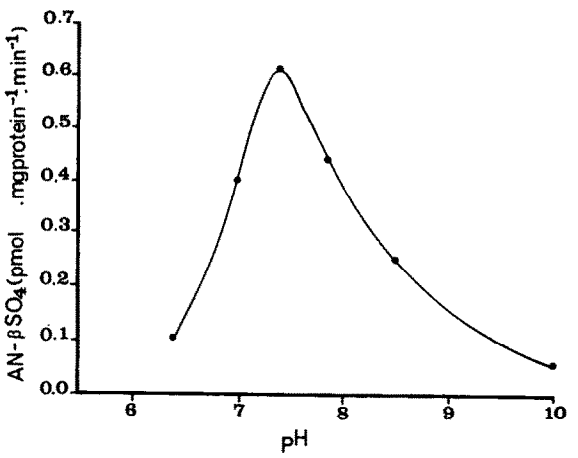

Fig.2. Microsomes were prepared and incubated with [5α,6α-^{3}H]an-β in buffer of varying pH, for 60 min as in section 2. The an-β sulphate was quantified and the optimum pH determined. pH 7.4 was employed for all further investigations.

those shown in table 1, when 2 mmol/l ATP were added. In the presence of 8 mmol/l ATP the reaction was inhibited to the extent of 60% (0.05 pmol an-β sulphate.mg protein^{-1} . min^{-1}). Inhibition of sulphokinase activity by ATP has been observed [18] although the inhibition found in that study was 20%.

3.3. *Rate of an-β sulphate synthesis by the microsomal fraction*

Table 2 shows that an-β sulphate was not obtained in appreciable quantities for the first 45 min of the assay. Thereafter, the quantities produced were much greater. Such biphasic kinetics were observed [18] for steroids which were considered less preferred by the enzyme system. To explain these kinetics it was suggested [18] that the formation of the 'active sulphate' is reversible and by rearranging the rate equation accordingly, it was shown that this could account for the lag phase observed. Alternatively, here an endogenous substrate may be metabolised in preference to an-β, and thus an-β sulphate would not be produced until the preferred substrate had been exhausted. In a preliminary study this microsomal preparation was shown to produce more DHA sulphate from DHA than an-β sulphate from an-β, and this would be in keeping with [15] where 5-ene-3β-hydroxy steroids were the preferred substrates for sulphokinase activity.

Table 2
Progress of an-β sulphate biosynthesis

Incubation (min)	An-β sulphate produced (pmol/mg protein)
0	0.0
30	0.235
45	0.0
60	1.81
75	3.63

Microsomal incubations were terminated at times indicated to ascertain the rate of the reaction. An-β sulphate was quantified as in section 2

3.4. *Solubilisation of microsomal sulphokinase activity*

Sonication (7 μm peakwidth) of pig liver microsomes for 2 min followed by ultracentrifugation (176 000 × *g*) yielded no solubilised sulphokinase. Similarly shaking buffered microsomal suspensions in Lubrol WX, Brij 35, Triton X-100, Nonidet LE and Tween 20 (0.5% solutions, pH 7.4) also failed to provide a solubilised and active enzyme system. However, sodium dodecyl sulphate (SDS) was capable of releasing an active an-β sulphokinase from the microsomes, 0.5% SDS (w/v) providing the most active preparation (table 3). The loss of enzyme activity caused by 1% SDS (w/v) (table 3) may be due to disaggregation of the component proteins, or to alterations in the structures of the individual proteins. Control experiments showed that an-β sulphate could not be formed by the direct action of SDS on the parent steroid.

To date investigations into sulphokinase activity have found that the enzyme system is cytosolic [14–16]. The solubilisation of the sulphokinase from

Table 3
Solubilization of an-β sulphokinase from porcine liver microsomes

SDS (g/l)	An-β sulphokinase activity (pmol. mg protein^{-1} . min^{-1})
0	0.04
1	0.40
5	0.81
10	0.39

Microsomes were shaken for 60 min in solutions of buffered SDS of varying concentrations. The mixture was centrifuged at 176 000 × *g* to remove particles and the clear supernatant assayed for an-β sulphokinase as in section 2

microsomes by SDS may be a consequence of the ionic strength of the medium, releasing loosely adsorbed proteins from the microsomes. This could explain the failure of the non-ionic detergents to release the enzyme system from microsomal membranes. Alternatively, the enzyme system may be firmly bound to the microsomes and therefore only SDS would solubilise the system. It is hoped that the properties of the cytosolic sulphokinase system will be investigated and compared to those of the microsomal system.

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