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STUDIES ON A 3β-HYDROXYSTEROID SULPHOTRANSFERASE FROM RAT LIVER

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Summary

A steroid sulphotransferase (EC 2.8.2.2) was partially purified from female rat liver. The enzyme was active towards the substrates, dehydroepiandrosterone, epiandrosterone and pregnenolone but was inactive towards oestrogens, cholesterol and ergocalciferol. A pH optimum of 5.0 was recorded but the enzyme was unstable at low pH. The enzyme was stimulated slightly by the addition of reducing agents and inhibited by *p*-chloromercuribenzoate and $HgCl_2$. Crude enzyme activity was markedly stimulated by divalent cations but this effect was not observed with purified enzyme. A K_m of 13 μM was calculated for the donor substrate 3′-phosphoadenylyl sulphate and the acceptor substrate, dehydroepiandrosterone had a K_m value of 6 μM. The enzyme appeared to be highly susceptible to product inhibition by adenosine 3′,5′-diphosphate.

Introduction

The biological role of steroid sulphates is still largely a matter of speculation. The metabolism of these compounds has been extensively reviewed [1—4]. Some steroid sulphates, especially dehydroepiandrosterone sulphate, occur in large quantities in blood and are synthesised by many tissues. Foetal dehydroepiandrosterone sulphate is known to be an important precursor of placental oestrogen in pregnancy [5] but the role of dehydroepiandrosterone sulphate in the male and the non-pregnant female is not understood. Sulphotransferases catalyse the sulphurylation of steroids using 3′-phosphoadenylyl sulphate as sulphate donor. There have been several reports describing the properties of steroid sulphotransferases (EC 2.8.2.2) [6—14]. Adams and coworkers succeeded in isolating a pure enzyme from bovine adrenal gland which catalyses the sulphurylation of oestrogens but not other acceptors [8—12]. Other attempts to purify steroid sulphotransferases have met with little success

[6,13,14]. In the absence of purified enzymes the nature, function and multiplicity of these enzymes remain obscure. The present paper describes the partial purification from rat liver of a 3β-hydroxysteroid sulphotransferase which catalyses the sulphurylation of dehydroepiandrosterone and pregnenolone but not oestrogens or cholesterol. Several of the enzymes' properties are discussed including evidence of its ability to exist in various molecular forms.

Experimental

Materials. Female rats of the Wistar strain, 3—6 months old were used throughout. Purification procedures were carried out at 0—4°C unless otherwise specified.

Preparation of 3'-phosphoadenylyl [^{35}S] sulphate

This was prepared essentially according to the method of McEvoy and Carroll [15]. The incubation mixture for the synthesis of 3'-phosphoadenylyl [^{35}S]-sulphate contained in a total volume of 40 ml: 4.2 mmol Tris · HCl buffer, pH 7.4, 200 μmol K_2SO_4, 240 μmol ATP, 2 mg creatine phosphokinase (Type 1, Sigma Chemical Co, U.K.), 50 mg phosphocreatine, 50 mCi $Na_2{}^{35}SO_4$ (carrier-free code SJSI, The Radiochemical Centre, U.K.), 60 μmol $MgCl_2$ and 16 ml high-speed (100 000 × *g*) supernatant fraction from a rat liver homogenate in four volumes 0.15 M KCl. The mixture was incubated with shaking, at 37°C for 2 h and the 3'-phosphoadenylyl [^{35}S] sulphate was isolated by chromatography on ECTEOLA cellulose (Bio-Rad Laboratories, Richmond, Calif.) according to the method of Balasubramanian et al. [16]. The yield of 3'-phosphoadenylyl [^{35}S] sulphate was 90 μmol with a specific radioactivity of 4.8×10^5 dpm/nmol.

Assay of steroid sulphotransferase activity

A micro, radioisotope assay was employed using 3'-phosphoadenylyl [^{35}S]-sulphate as the radioactive substrate. The incubation mixture contained in a total volume of 40 μl: 2 μmol Tris · HCl buffer, pH 8.0, 2 nmol dehydroepiandrosterone in 10 μl of propylene glycol, 10 nmol 3'-phosphoadenylyl [^{35}S]-sulphate and 10—100 μg protein. Incubation took place at 37°C and the reaction was terminated by immersion of the reaction tubes in boiling water for 60 s. Enzymic activity was determined by measurement of the radioactive steroid sulphate formed. 20-μl aliquots of the reaction mixture were applied to Whatman No. SG81 silica gel-impregnated paper and chromatographed for 3 h using the solvent chloroform/methanol/water (65 : 25 : 4, by vol.). In this system the sulphurylated steroids migrate almost with the solvent front while the sulphurylated nucleotides and inorganic sulphate remain at the origin. The developed chromatograms were sectioned and the regions containing steroid sulphate were placed in scintillation vials to which 10 ml of scintillation fluid (0.03% POPOP and 0.5% PPO in toluene) were added. Radioactivity was measured using a Packard (Model 3375) scintillation counter and enzymic activity expressed as nmol ester sulphate formed/mg protein during 5 min incubation. Sulphotransferase activity towards water-soluble acceptor compounds was assayed according to the method of McEvoy and Carroll [15]. The protein content of solutions was determined by the method of Lowry et al. [17].

Preparation of adenosine 3',5'-diphosphate

3'-Phosphoadenylyl sulphate was hydrolysed using 0.1 M HCl for 30 min at 37°C and the resulting adenosine 3',5'-diphosphate was isolated by chromatography on Dowex AG1-X-2 according to the method of Cramer et al. [18].

Isoelectric focusing

The apparatus and reagents were purchased from LKB-Produckter AB, Sweden. A 110 ml column (LKB 8101) was used and the experimental procedure adopted was according to the LKB Ampholine Instruction Manual (1973) 1-8100-E02. The cathode was located at the top of the column.

Enzyme purification procedure

Stage I. Rats were killed by cervical dislocation and the livers were placed in cold isotonic KCl solution. In a typical purification 56 g of liver were homogenized in 280 ml of cold 0.15 M KCl using a Potter-Elvehjem homogenizer and the suspension was centrifuged for 1 h in an MSE-75-Superspeed centrifuge at 100 000 × *g*. Clear supernatant was collected and this solution was diluted using 0.15 M KCl to a protein concentration of 10 mg/ml.

Stage II. Cold ethanol was added with stirring to the diluted supernatant fluid from Stage I until the concentration of ethanol was 25%. This solution was stirred at −10°C for 20 min after which precipitated protein was collected by centrifugation at −10°C. The supernatant fraction was discarded and the precipitate was dissolved in 0.01 M Tris · HCl buffer, pH 8.0, containing 10% glycerol and dialysed overnight against 10 l of the same buffer. The solution was clarified by brief centrifugation at 10 000 × *g*.

Stage III. The sulphotransferase preparation from Stage II was applied to a DE-52 cellulose column (2.1 × 26.5 cm) previously equilibrated with 0.01 M Tris · HCl buffer, pH 8.0, containing 10% glycerol. After washing with the equilibrating buffer to remove unbound protein the column was eluted using a linear salt gradient generated using 400 ml of 0.01 M Tris · HCl buffer containing 10% glycerol in one vessel and a reservoir containing 400 ml of 0.4 M KCl in 0.01 M Tris · HCl buffer, pH 8.0, containing 10% glycerol. This was followed by irrigation with 1 M KCl in 0.01 M Tris · HCl buffer, pH 8.0, containing 10% glycerol which removed additional protein with no detectable sulphotransferase activity. Enzyme and protein elution profiles are illustrated in Fig. 1. The most active fractions were pooled and concentrated by ultrafiltration through a UM-10 membrane (Amicon Corp.). The concentrated protein solution was dialysed overnight against two times 5-l quantities of 0.01 M citrate buffer, pH 6.2, containing 10% glycerol and clarified by centrifugation at 10 000 × *g*.

Stage IV. The enzyme solution from Stage III was applied to a cellulose phosphate (P11 Whatman) column (25 × 1.3 cm) previously equilibrated with 0.01 M citrate, pH 6.2, containing 10% glycerol. The column was irrigated with equilibrating buffer which displaced a large inactive protein fraction. The sulphotransferase was then eluted from the column using a linear salt gradient generated using 250 ml of 0.01 M citrate buffer, pH 6.2, containing 10% glycerol in one vessel and a reservoir containing 250 ml of 0.5 M KCl in 0.01 M citrate buffer, pH 6.2, containing 10% glycerol. A number of protein peaks

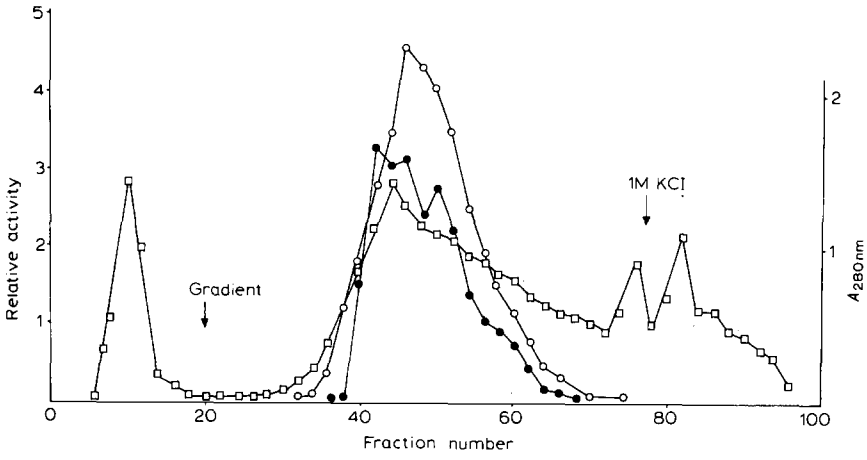

Fig. 1. Chromatography of enzyme from Stage II on DEAE-cellulose. 15-ml fractions were collected and the elution of sulphotransferase activity towards dehydroepiandrosterone (●———●) and *p*-nitrophenol (○———○) as acceptor substrates is plotted. The absorbance (□———□) of the fractions at 280 nm is shown.

were obtained (Fig. 2) and sulphotransferase activity was separated from the major protein fractions. The active sulphotransferase fractions were pooled, adjusted to pH 8.0 by titration with 1 M Tris · HCl, pH 8.0, and concentrated by ultrafiltration.

Stage V. The partially purified sulphotransferase was subjected to further purification by isoelectric focusing prior to the investigation of the substrate specificity of the enzyme. Electrofocusing was carried out at an ampholyte concentration of 1%, pH range 3—10, stabilised by a sucrose gradient and cooled by circulating ethanol solution at 0°C. 10 ml of enzyme from Stage IV were dialysed against 3 l of 4% glycine for 4 h prior to focusing. A power input of approx. 2 W was maintained during focusing and the current was minimal after 40 h. 1-ml fractions were collected and assayed for sulphotransferase

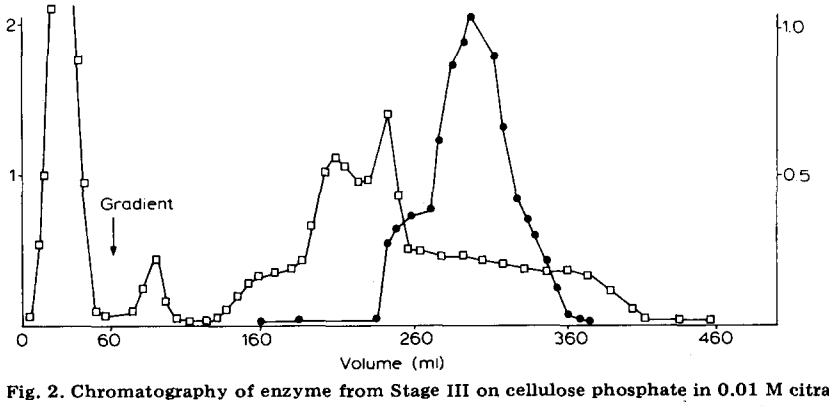

Fig. 2. Chromatography of enzyme from Stage III on cellulose phosphate in 0.01 M citrate buffer, pH 6.2, containing 10% glycerol. The elution of sulphotransferase activity (●———●) towards dehydroepiandrosterone and the absorbance (□———□) of the fractions at 280 nm are shown.

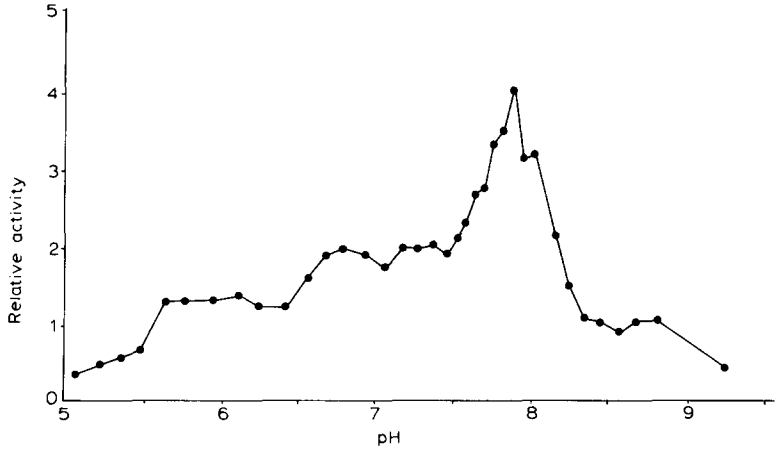

Fig. 3. The behaviour of sulphotransferase activity towards dehydroepiandrosterone on electrofocusing. The enzyme from Stage IV of the purification was subjected to a power input of approx. 2 W for 40 h in a 110 ml column of ampholine pH range 3—10.

activity towards dehydroepiandrosterone as acceptor substrate. 52% of the applied enzymic activity was recovered but this was spread over a wide range of pH in four apparently distinct zones of activity (Fig. 3). 24% of the applied activity was recovered in the fourth peak (pH 7.5—8.3) and this was pooled and dialysed against 10 l of 0.01 M Tris · HCl, pH 8.0. The concentration of protein in this fraction (Stage V enzyme) was below the level of accurate measurement and the specific activity of this enzyme fraction was not determined.

Further isoelectric focusing studies

Refocusing of the Stage V enzyme under conditions identical with those used in the isolation of this fraction from the Stage IV enzyme again gave rise to multiple peaks of dehydroepiandrosterone sulphotransferase activity. Enzymic activity was distributed over the range pH 5.5—8.5 with peaks of activity at pH 5.85, 6.75, 7.30 and 7.90. The relative activities of the four peak fractions were in approximately the same ratio to each other as found in the electrofocusing of Stage IV enzyme. The fractions eluted over the range pH 5.5—6.1 were combined and refocused over the range pH 3—10 for 40 h. The eluted dehydroepiandrosterone sulphotransferase activity was again found to be dispersed over the range pH 5.5—8.5 with four peaks of activity with apparent p*I* values identical with those found in the electrofocusing of enzyme from Stages IV and V of the purification.

Results

A summary of the purification of the sulphotransferase following precipitation by ethanol and chromatography on DEAE- and phospho-cellulose is presented in Table I: an approx. 60-fold enrichment of sulphotransferase activity employing dehydroepiandrosterone as acceptor substrate was achieved. The specific activity of Stage V enzyme was not determined.

TABLE I

SUMMARY OF THE PARTIAL PURIFICATION OF A STEROID SULPHOTRANSFERASE FROM RAT LIVER SUPERNATANT

Specific activity is expressed as nmol dehydroepiandrosterone [^{35}S]sulphate formed/mg protein per 5-min incubation.

Stage		Volume (ml)	Total protein (mg)	Recovery of activity (%)	Specific activity
I	High speed supernatant	270	4252.5	100	1.48
II	Ethanol precipitation (0—25%)	104	1430	86	3.78
III	DEAE-cellulose chromatography	76.5	125.5	28	13.86
IV	Phosphocellulose chromatography	60	6.3	8.6	85.8

Properties of the purified sulphotransferase

Sulphotransferase activity towards dehydroepiandrosterone as acceptor substrate was stable for several months at −20°C but declined by 60% after 4 weeks at 4°C.

The influence of pH on the activity of the enzyme was determined using a series of buffers in the range pH 4—9. The buffers were prepared by titrating with 0.2 M NaOH a solution containing citric acid, potassium dihydrogen phosphate, orthoboric acid and diethylbarbituric acid each at a concentration of 28.6 mM. The enzyme solution was dialysed against distilled water for 4 h prior to its assay: optimum activity at pH 5 was recorded (Fig. 4a). The effect of pH on the stability of the enzyme was investigated by preincubating the enzyme with the same series of buffers for 30 min at 37°C: the enzyme solutions were then cooled in ice and brought to pH 8 by adding an equal volume of 0.2 M Tris · HCl buffer, pH 8. The enzyme was then assayed over a 5-min incubation period at pH 8 and the results are presented in Fig. 4b.

Added divalent cations, especially Fe^{2+}, Co^{2+} and Mn^{2+}, greatly increased (by up to 100%) enzymic activity of the crude enzyme fraction, but neither the addition of EDTA nor these cations at concentrations up to 5 mM had effect on the activity of the purified enzyme. The activity of the purified enzyme was enhanced slightly by the addition of reduced glutathione and was inhibited to the extent of 80% by the presence of 0.1 mM $HgCl_2$.

Kinetics of dehydroepiandrosterone sulphotransferase

During incubation at 37°C, the activity of the purified enzyme was linear with time for a period not exceeding 5 min; the decline in activity after that time was not due to substrate limitation. The effects of varying substrate concentration on the activity of the enzyme were determined and initial velocities were plotted according to the method of Lineweaver and Burk [19]. Substrate inhibition at high concentrations of either the donor (3′-phosphoadenylyl sulphate) or the acceptor substrate (dehydroepiandrosterone) was observed: Asymptotes were drawn to the double reciprocal plots (Figs. 5 and 6) and a

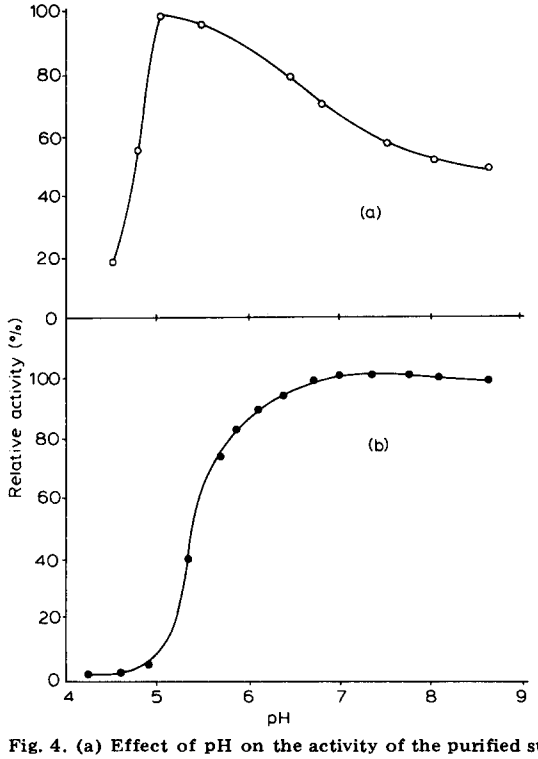

Fig. 4. (a) Effect of pH on the activity of the purified sulphotransferase using dehydroepiandrosterone as acceptor substrate. The buffer employed in the assay consisted of a solution containing citric acid, potassium dihydrogen phosphate, orthoboric acid and diethylbarbituric acid, each at a concentration of 28.6 mM and triated to the required pH with 0.2 M NaOH. Enzymic activity was measured over a 5-min period at 37°C. (b) The effect of pH on the stability of the purified 3β-hydroxysteroid sulphotransferase. The enzyme was preincubated for 30 min at 37°C with the buffers used in (a) and then brought to pH 8.0 by the addition of 0.2 M Tris · HCl buffer. Enzymic activity at pH 8.0 was then determined over a 5-min incubation period.

converging pattern was evident. Intercepts and slopes from Figs. 5 and 6 were plotted against the reciprocal of the appropriate second substrate and the K_m values obtained from these graphs for 3′-phosphoadenylyl sulphate and dehydroepiandrosterone were 13 and 6 μM, respectively.

Adenosine 3′,5′-diphosphate, a product of the reaction, inhibited sulphotransferase activity: with 3′-phosphoadenylyl sulphate as the variable substrate and the concentration of dehydroepiandrosterone non-saturating (2.6 μM), adenosine 3′,5′-diphosphate was a competitive inhibitor with a K_i of approx. 3 μM.

Substrate specificity

A wide range of steroid and non-steroid compounds were tested as possible acceptor substrates for the purified enzyme. The relative activities obtained employing a selection of these substrates are summarized in Table II and compared to results obtained with the crude enzyme. Maximum activity was observed when the steroid was of the allo-series and possessed a 3β-hydroxyl and 17-oxo function.

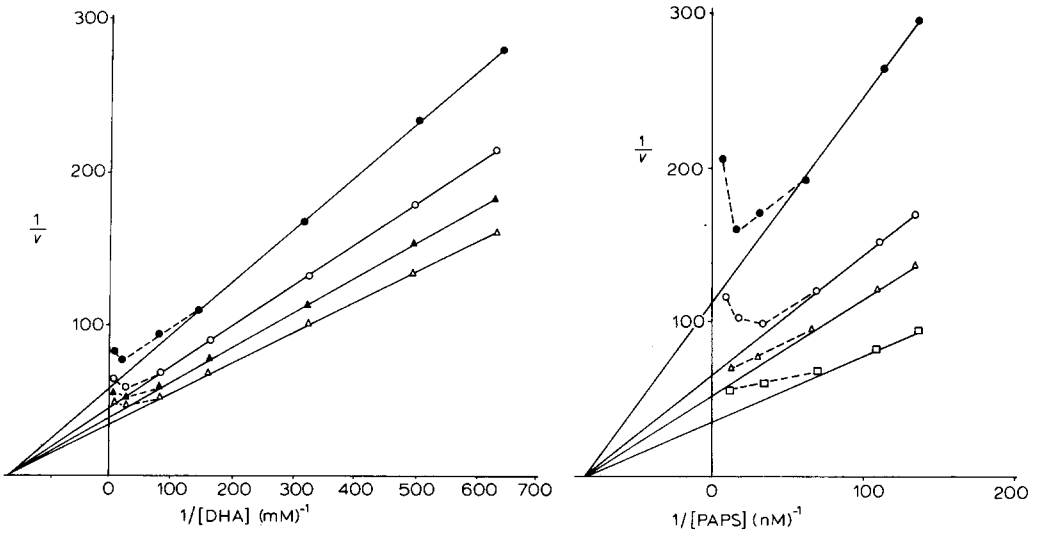

Fig. 5. The effect of dehydroepiandrosterone (DHA) concentration on sulphotransferase activity at four different concentration of the donor substrate: the concentrations of 3′-phosphoadenylylsulphate were 7.4 μM (●———●), 14.8 μM (○———○), 29.6 μM (▲———▲) and 59.2 μM (△———△).

Fig. 6. The effect of 3′-phosphoadenylylsulphate (PAPS) concentration on sulphotransferase activity towards dehydroepiandrosterone at four different concentrations of the acceptor substrate. The concentrations of dehydroepiandrosterone were 1.55 μM (●———●), 3.1 μM (○———○), 6.2 μM (△———△) and 12.4 μM (□———□).

Gel filtration studies

1 ml of a solution of the partially purified (Stage IV) enzyme was chromatographed on a column (1.2 × 98 cm) of Biogel A 1.5 M (200–400 mesh) (Biorad Laboratories, Richmond, Calif.) previously equilibrated with 0.15 M KCl in

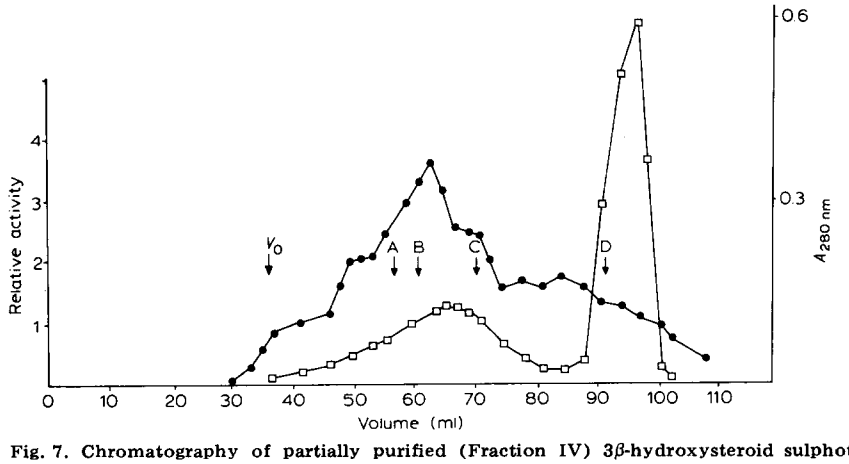

Fig. 7. Chromatography of partially purified (Fraction IV) 3β-hydroxysteroid sulphotransferase on a column (1.2 × 98 cm) of Biogel A 1.5 M in 0.05 M Tris · HCl buffer, pH 8.0, and containing 0.15 M KCl. The relative activity (●———●) and absorbance at 280 nm (□———□) of the eluted fractions are plotted. The void volume of the column (V_0) and the positions of elution of catalase (A), yeast alcohol dehydrogenase (B), peroxidase (C) and cytochrome *c* (D) are also shown.

TABLE II

RELATIVE ACTIVITY OF STAGE V AND UNFRACTIONATED RAT LIVER SULPHOTRANSFERASE TOWARDS STEROID AND OTHER ACCEPTOR SUBSTRATES

Activity is expressed as a percentage of the initial rate of sulphurylation of the substrate relative to the initial rate of synthesis of dehydroepiandrosterone sulphate.

Acceptor substrate	Relative activity	
	Purified enzyme	Crude enzyme
Dehydroepiandrosterone	100	100
Epiandrosterone	137	140
5α-Pregnane-3β,20α-diol	86	—
5α-Androstane-3β,17β-diol	83	—
Androst-5-ene-3β,17β-diol	66	—
Pregnenolone	61	57
Androsterone	53	46
3β-Hydroxy-5β-androstan-17-one	45	—
5α-Androstane-3α,17β-diol	20	—
11-Deoxycorbicosterone	10	30
5β-Androstane-3α,17β-diol	7	—
p-Nitrophenol	8	93
L-Tyrosinemethyl ester	9	92
Butan-1-ol	7	14
Testosterone	2	14
Corticosterone	6	—
3β,17α-Dihydroxy-pregn-5-ene-20-one	4	—
α-Naphthol	4	44
3α-Hydroxy-5β-androstan-17-one	5	—
Oestrone	0	12
Oestradiol	0	—
Cholesterol	0	—
Cortisol	0	18
11β-Hydroxy-pregn-4-ene-3, 20-dione	0	—
Ergocalciferol	0	—
β-Naphthol	0	—
α-Naphthylamine	0	27
Bilirubin	0	—
Diethylstilbestrol	0	—

0.05 M Tris · HCl buffer, pH 8. The column effluent was collected in 1-ml fractions and these were assayed for sulphotransferase activity using dehydroepiandrosterone as acceptor substrate: the pattern of elution of this activity is shown in Fig. 7. 1-ml samples of solutions of Blue Dextran 2000 (Pharmacia, Sweden) and of commercially prepared (Sigma Chemical Co., U.K.) samples of catalase, yeast alcohol dehydrogenase, peroxidase and cytochrome *c* were also chromatographed on the same column of Biogel A 1.5 M. The exclusion volume of the column and the volumes corresponding to the peaks of the elution profiles of these proteins are also shown in Fig. 7.

Discussion

The properties of the 3β-hydroxysteroid sulphotransferase described in this paper have some similarities with those of other known sulphotransferases: an

exception is the lower pH optimum recorded in this present case. The kinetic data obtained is consistent with the existence of a random mechanism. Substrate inhibition by dehydroepiandrosterone at low concentrations of 3′-phosphoadenylyl sulphate was observed. This is in agreement with the findings of Adams and Edwards [13] in their studies on the sulphurylation of dehydroepiandrosterone by human adrenal extracts. The K_m of this 3β-hydroxysteroid sulphotransferase from rat liver for dehydroepiandrosterone (K_m = 6 μM) is the same as that calculated by Gugler and Breuer [14] for a steroid sulphotransferase isolated from human liver.

An important feature of the kinetic properties of the rat liver 3β-hydroxysteroid sulphotransferase is its susceptibility to inhibition by adenosine 3′,5′-diphosphate. Consequently, the changing concentration of this product of the sulphotransferase reaction during the assay period, must be acknowledged in interpreting data from kinetic studies. The apparent stimulatory effect of divalent cations on this and other sulphotransferases in the crude but not in the purified state may be an artifact of the assay system: added cation may be stimulating the activity of the 3′-nucleotidase which preferentially degrades adenosine 3′,5′-diphosphate over 3′-phosphoadenylyl sulphate [20—22]. Conversely, the apparent difficulty of achieving substantial enrichment of the specific activity of the sulphotransferases in this and other studies may be a consequence of the gradual removal of adenosine 3′,5′-diphosphate hydrolyses from the sulphotransferase, thus allowing the accumulation of this inhibitor during the assay.

A major obstacle to an understanding of the importance and roles of the sulphotransferases has been the lack of data on the precise number of these enzymes which exist in the various tissues. This problem is particularly relevant to the liver which has unequalled versatility in sulphurylating activity. It is clear that a distinct phenolsulphotransferase exists [6,15,28]. There is evidence [23] for the separate existence of a tyrosine methyl ester sulphotransferase. With regard to steroid sulphotransferases, Banerjee and Roy [6] and Nose and Lipmann [24] gave evidence that an enzyme active towards dehydroepiandrosterone was distinct from one catalysing the sulphurylation of oestrone in guinea pig and rabbit liver. Adams and coworkers [8—12] have isolated an oestrogen sulphotransferase which has no activity towards dehydroepiandrosterone and simple phenols. Distinct sulphotransferases have also been proposed for the steroids 11-deoxycorticosterone and 3α-hydroxy-5β-androstan-17-one. The sulphurylation of vitamin D [26] and cholesterol [7] is thought to be catalysed by dehydroepiandrosterone sulphotransferase. In the present study, comparison of the relative rates of sulphurylation of a large range of compounds by the crude and the partially purified enzyme indicates that: this enzyme catalyses the sulphurylation of dehydroepiandrosterone, epiandrosterone, pregnenolone and androsterone; this enzyme is distinct from the sulphotransferase active towards cholesterol, vitamin D, 11-deoxycorticosterone, oestrogens, testosterone and several non-steroid substrates; a separate sulphotransferase exists for steroids of the 5β series. This is supported by the observation of a low rate of sulphurylation of 3α-hydroxy-5β-androstan-17-one which, in human liver, is sulphurylated at the same rate as dehydroepiandrosteron [27].

Several of the sulphotransferases have been shown to associate into polymeric

forms [13,15,28]. Gel filtration studies on the crude and partially purified (Stage IV), 3β-hydroxysteroid sulphotransferase isolated in the present study shows that this enzyme does not chromatograph as a single molecular species: rechromatography on Biogel A 1.5 M of individual fractions from the chromatogram shown in Fig. 7 again gave rise to an apparent mixture of 3β-hydroxysteroid sulphotransferase activities with diverse molecular size. The heterogeneity of the purified sulphotransferase is shown also by its behaviour on isoelectric focusing.

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