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ENZYMIC SYNTHESIS OF STEROID SULPHATES

IX. PHYSICAL AND CHEMICAL PROPERTIES OF PURIFIED OESTROGEN SULPHOTRANSFERASE FROM BOVINE ADRENAL GLANDS, THE NATURE OF ITS ISOENZYMIC FORMS AND A PROPOSED MODEL TO EXPLAIN ITS WAVE-LIKE KINETICS

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SUMMARY

A method is described for the preparation of oestrogen sulphotransferase (3′-phosphoadenylylsulphate:oestrone sulphotransferase, EC 2.8.2.4) in pure form from bovine adrenal glands. Analytical ultracentrifuge studies gave $s^0_{20,w} = 4.92$. The molecular weight, as determined by centrifugation methods, was 74 000 ± 700. No evidence of the presence of subunits, or of an association–dissociation system, could be found. The presence of substrates had no effect on the sedimentation properties.

After reduction and alkylation, 23 residues of *S*-carboxymethyl cysteine per mole were found. In the native enzyme only 0.2 SH groups per mole could be titrated; this value being increased to approximately 1.0 upon addition of sodium dodecylsulphate. Addition of thiols resulted in activation. Dithiothreitol activated when low oestrogen levels were used, but inhibited when high estrogen levels were used in assays. *p*-Hydroxymercuribenzoate, at 0.1 mM, was inhibitory only when the enzyme was assayed at low oestrogen concentrations. Higher concentrations (0.5 mM) inhibited at all oestrogen levels.

Wave-like kinetics were obtained when oestrogen was varied at constant 3′-phosphoadenylylsulphate but normal Michaelis–Menten kinetics were obtained when 3′-phosphoadenylylsulphate was the variable substrate. Inhibition studies with adenine nucleotides indicated that both 3′- and 5′-phosphate groups are probably involved in the binding of the substrate to the enzyme.

Four isoenzymes have been isolated and their properties compared. Each isoenzyme consists of a single polypeptide chain of molecular weight about 74 000. Amino acid analyses were similar, but significant differences were observed particularly in Lys, Asp, His and Met. The specific activities differed and partial resolution of the isoenzymes, which occurred upon chromatography on ion-exchange columns,

Abbreviations: *P*-Ado-*PS*, adenosine-3′-phosphate-5′-phosphosulphate; *P*-Ado-*P*, adenosine-3′-phosphate-5′-phosphate.

resulted in the formation of multi-peaks in the elution profiles. Each isoenzyme exhibited wave-like kinetics on varying oestrogen at constant 3′-phosphoadenylylsulphate.

The existence of a number of enzyme conformers is proposed which differ in the accessibility of the single SH group and in their affinity for oestrogen; the existence of these low- and high-K_m species then accounting for the wave-like kinetics exhibited by the enzyme.

INTRODUCTION

Sulphated forms of steroids are now under increasing scrutiny due to their known involvement in steroid interconversions, their secretion from the adrenal gland and their high concentration in blood [1]. Oestrone sulphate is the major oestrogen in the blood in the human female and has been found to be in equilibrium with free oestrone and 17β-estradiol in the blood [2]. Sulphate conjugation can be important in certain aspects of oestrogen metabolism, since, for example, oestrone-3-sulphate is reduced at the C_{17}-position to a greater extent than free oestrone by liver homogenates [3]. Other evidence has shown that the oestrogen metabolite 2-methoxyoestrone is derived from oestrone-3-sulphate; the sulphate group being obligatory for the 2-hydroxylation to occur [4]. More recently, the importance of steroid sulphotransferase enzyme levels in human breast tumours, in the prediction of both prognosis and response to hormone ablative procedures, has been emphasised [5].

Little progress has been made in the purification of steroid alcohol sulphotransferases, but an enzyme which sulphurylates the natural estrogens, but not simple phenols or steroid alcohols, has been previously described [6–8]. The enzyme possessed some unusual features: peaks and throughs being obtained in the kinetic curves when oestrogen was varied at constant adenosine-3′-phosphate-5′-phosphosulphate (*P*-Ado-*PS*) [7]. Pure enzyme has now been isolated, its chemical and physical properties described and a model proposed to explain the unusual kinetic features. The nature of the isoenzymic forms is also reported.

MATERIALS

Adenosine-3′-phosphate-5′-phospho-[^{35}S]sulphate (*P*-Ado-*P*^{35}*S*) was prepared as described previously [6]. Unlabelled *P*-Ado-*PS* was prepared enzymically by the method of Brunngraber [9], except that precipitation of the mercuri complex was omitted: the incubation mixture was placed in a boiling water bath for 1 min and after dilution the supernatant was chromatographed on Dowex-1X8-400 resin in the Cl^- form. The purity of labelled and unlabelled *P*-Ado-*PS* was checked at intervals by paper electrophoresis in sodium phosphate buffer, pH 6.0.

Adenosine-3′-phosphate-5′-phosphate (*P*-Ado-*P*) was prepared by incubation of *P*-Ado-*PS* in 0.1 M HCl at 37 °C for 30 min followed by neutralisation. Other nucleotides were obtained from Sigma Chemical Co. Enzyme grade $(NH_4)_2SO_4$ was obtained from Schwarz/Mann and used for enzyme purification. *p*-Hydroxymercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid), and oestrone were obtained from Sigma

Chemical Co. L-Cysteine hydrochloride and dithiothreitol were from Calbiochem. DEAE-cellulose (DE11 and DE32) were Whatman products and were treated according to the manufacturer's instructions. Bovine serum albumin (Sigma) was run through new columns to saturate irreversible protein-binding sites.

Anilino-8-naphthalene sulphonic acid was an Eastman product. Maleic anhydride and sodium dodecylsulphate were obtained from Sigma. Coomassie brilliant blue R250 was obtained from I.C.I. and Amidoschwartz 10B from Merck. The sources of the proteins used were as follows: wheat germ lipase (Sigma), phosphorylase, ovalbumin and chymotrypsinogen (Mann Research), trypsin (Worthington Biochemical Corp.). Protein phosphate phosphorylase was a gift from Dr A. Mackinlay.

METHODS

Protein was determined by the method of Lowry et al. [10] using crystalline bovine serum albumin (Sigma) as reference protein.

Liquid scintillation counting was carried out on a Packard Tricarb 2022 instrument using the phosphor mixture as previously described [6].

Standard enzyme assay

Oestrogen sulphotransferase activity was measured in the following incubation medium: 0.15 mM *P*-Ado-*PS*; 10^5 cpm *P*-Ado-$P^{35}S$; 0.05 mM oestrone added as 5 μl of a propyleneglycol stock solution; 15 mM $MgCl_2$; 0.1 M Tris–HCl buffer, pH 7.5; enzyme 10–50 μg. Total volume was 0.15 ml. Incubation was carried out for 20 min at 37 °C. Any departures from these conditions are stated. The assay procedure was as previously described [6].

Standard enzyme preparation

Freshly collected and trimmed bovine adrenal glands (500 g) were homogenised with 2 vol. of 0.01 M phosphate in 0.9% saline (pH 7.4) employing a Waring Blendor. The homogenate was centrifuged at 13 000 $\times$ *g* for 15 min to remove cell debris and then at 40 000 $\times$ *g* for 1 h. The supernatant which contained about 20 mg protein per ml was then treated with $(NH_4)_2SO_4$ and the fraction precipitating between 0.5 and 0.7 saturation collected, dissolved in 50 ml of 0.02 M phosphate buffer, pH 7.8, and dialysed extensively against the same buffer (3 $\times$ 8 l). The preparation was placed on a 4 cm $\times$ 40 cm column of DEAE-cellulose (DE11) which had been equilibrated with 0.02 M phosphate buffer, pH 7.8. Elution was commenced with this buffer at a flow rate of 8 ml/h per cm^2 and 12-ml fractions collected. After the red haemoglobin had been eluted, the eluting buffer was changed to 0.06 M phosphate, pH 7.8. Fractions (0.1 ml) were assayed for enzyme activity and the pooled active fractions concentrated by vacuum dialysis and then dialysed against 0.02 Mphosphate buffer, pH 7.8.

The preparation (about 40 ml) was then placed on a 2.5 cm $\times$ 25 cm column of DEAE-cellulose (DE32) and eluted with a linear gradient 0.02–0.1 M phosphate, pH 7.8 (400 ml). The flow rate was 8 ml/h per cm^2 and 6-ml fractions collected. Active fractions (see Fig. 1b) were pooled, concentrated by vacuum dialysis, dialysed against 0.1 M Tris–HCl buffer, pH 7.5, and stored either at 0 °C under N_2, or frozen in ampoules at −20 °C.

Amino acid analysis

Enzyme (5 mg) was dissolved in 8 M urea (5 ml), mercaptoethanol (0.04 ml) added and the pH of the solution raised to 10.5 with 5 M KOH. After standing under N_2 for 3 h, iodoacetic acid (134 mg) in 3 M Tris–HCl buffer, pH 8.5 (0.4 ml) was added and after standing for 10 min, the solution was dialysed against distilled water and lyophilised. *S*-Carboxymethyl enzyme (2 mg) was hydrolysed in 6 M HCl containing phenol (1 mg/ml) for 24 h at 110 °C. Amino acid analysis was carried out on a Beckman 102C instrument and corrections made for destruction of serine and threonine. Samples of isoenzymes, isolated from acrylamide gel slabs, were hydrolysed without prior reduction and alkylation.

Determination of sulphydryl and disulphide groups

Protein sulphydryl groups were determined by the methobs of Ellman [11] and Boyer [12]. The method of Zahler and Cleland [13] was used for the assay of disulphide groups.

Sedimentation studies

A Beckman Model E analytical ultracentrifuge was employed. Details of sedimentation velocity and sedimentation equilibrium runs are given in the legends. For sucrose gradient studies, enzyme (200 μg) and yeast alcohol dehydrogenase (300 μg) in base buffer (0.3 ml), was layered on the gradients. The gradients (5–20%, w/v) were prepared in 0.05 M Tris–HCl, pH 9.0; 0.05 M sodium phosphate, pH 7.0, and 0.05 M sodium phosphate, pH 5.8, respectively. The SW41 rotor of the Beckman Centrifuge was used at 30 000 $\times$ *g* for 21 h. 19 fractions were collected and assayed for oestrogen sulphotransferase and alcohol dehydrogenase activities.

Isolation of individual isoenzymes

Small amounts of the isoenzymes were isolated by electrophoresis on 4% acrylamide gel slabs. Visualization of protein was achieved on guide strips immersed in 1 M HCl for 2 min and then in anilino-8-naphthalene sulphonic acid [14]. Recovery of protein and enzyme units by the above method was poor and resort was made to ion-exchange methods. Enzyme was purified to the first DEAE-cellulose column stage (above) and protein (50 mg in 5 ml of 0.02 M phosphate buffer, pH 7.8) was loaded onto a 2.2 cm $\times$ 18 cm column of DEAE-cellulose (DE32), equilibrated with the same buffer. Elution was carried out with a linear gradient of 0.02 M phosphate, pH 7.8, and 0.06 M phosphate, pH 7.0, at a flow rate of 15 ml/cm^2 per h and 3-ml fractions collected. These conditions differ from those used at the second column stage employed in the isolation of enzyme, as described under Standard enzyme preparation. Partial resolution of the isoenzymes occurred and by rechromatography of selected fractions on smaller columns, pure samples of isoenzymes 1–3 could be obtained. Acrylamide gel electrophoresis was used to analyse column fractions.

Maleylation

Protein was reduced and S-carboxymethylated by the method of Butler et al. [15]. Enzyme (10 mg) was dissolved in 6 M guanidine hydrochloride in 0.1 M sodium phosphate buffer, pH 8.0 (2 ml) and the tube flushed with N_2. Dithiothreitol (5 μmoles in 0.01 ml water) was added and the solution kept at room temperature

for 2 h. Iodoacetic acid (10 μmoles in 1 ml phosphate buffer, pH 8.0) was added and the tube again flushed with N_2. After 3 h, the solution was dialysed overnight against 6 M guanidine hydrochloride in 0.1 M sodium pyrophosphate. Maleic anhydride (10 mg) was added slowly and the pH adjusted to 8.5–9.0 with 1 M NaOH after each addition. The maleylated protein was then dialysed against 0.05 M Tris–HCl, pH 8.1.

Gel patterns of individual bovine adrenal glands

Adrenal glands from 27 individual animals were separately taken to the $(NH_4)_2SO_4$ fractionation stage of enzyme purification. The 0.55–0.7-saturated $(NH_4)_2SO_4$ fractions were dialysed against 0.05 M Tris–HCl, pH 8.0, and aliquots examined by electrophoresis on acrylamide slabs using 1% Amido Black in 7% acetic acid as the protein stain [16]. In addition, adrenal glands from 4 females and 3 male animals were processed through the first DEAE-cellulose column stage and aliquots of the pooled enzymically active fractions examined by electrophoresis on acrylamide slabs. The gels in these cases were stained with 0.05% Coomassie Brilliant Blue in 12.5% trichloroacetic acid and scanned on a Vitatron TLD densitometer using a 553 nm filter.

Acrylamide gel electrophoresis in sodium dodecylsulphate

The method of Weber and Osborn [17] was employed. Before electrophoresis, proteins were incubated at 37 °C for 2 h in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecylsulphate and 1% β-mercaptoethanol. The concentrations of the latter were then reduced to 0.1% by dialysis. Aliquots of protein (10–20 μg), together with chymotrypsinogen as protein marker, were loaded onto each gel. Electrophoresis was carried out for 6 h in 0.1 M phosphate buffer, pH 7.0, at a current of 8 mA per gel.

RESULTS

Standard purification procedure

The enzyme isolated by the method previously described, while specific for natural oestrogens and lacking activity for simple phenols and steroid alcohols, still contained a high percentage of contaminating protein [6]. The procedure finally adopted gave pure enzyme in yields of approximately 100 mg from 500 g of bovine adrenals. Fig. 1a shows the elution profile from the first DEAE-cellulose column and the corresponding acrylamide-gel pattern obtained with the pooled enzyme fractions. In addition to the four-band pattern which is associated with enzyme activity [7], a less polar and a more polar protein were present as contaminants. These were effectively removed on a fine-grade DEAE-cellulose column, employing a linear phosphate buffer gradient. By cutting and pooling the fractions as shown in Fig. 1b, pure enzyme, composed of four individual isoenzymes, was obtained. The preparation showed an absorption maximum at 280 nm; no absorption peaks being present at higher wavelengths which would indicate the presence of bound vitamin A [18]. The latter has been suspected of playing a role in sulphate activation and transfer. The absorbance ratio at 280/260 nm was 1.6 and the $E^{1\%}_{1cm}$ 8.7.

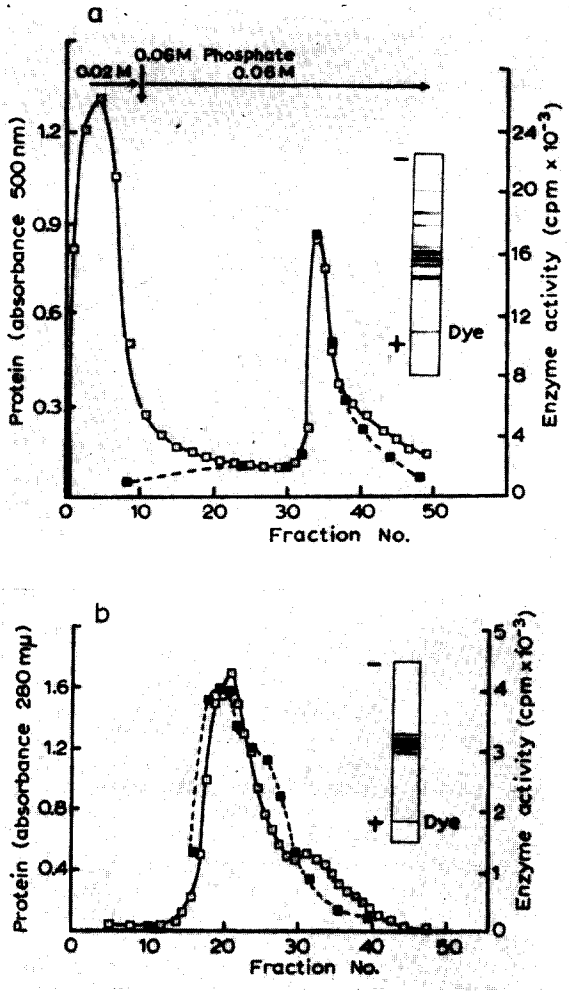

Fig. 1. a. Standard enzyme preparation: first DEAE-cellulose column. The 0.55–0.70 saturation $(NH_4)_2SO_4$ fraction was applied. Fractions were assayed for protein (□) and enzyme activity (■). An aliquot of the pooled enzyme peak (fractions 33–38) was analysed by acrylamide-gel electrophoresis. b. Standard enzyme preparation: second DEAE-cellulose column. The peak from Fig. 1a was applied to the column and eluted as described in Methods. Fractions were assayed for protein (□) and enzyme activity (■). An aliquot of the pooled enzyme (fractions 16–28) was studied by acrylamide-gel electrophoresis. The four-band pattern is shown which represents isoenzymes 1–4, in order of increasing negative charge.

Molecular weight studies

The purified enzyme centrifuged as a single boundary in sedimentation velocity experiments. Determination of the sedimentation coefficient at a number of protein concentrations in the range 1–10 mg/ml, showed it to be independent of protein concentration (Fig. 2a). Extrapolation of the sedimentation coefficient to infinite dilution gave a value of $s^0_{20,w} = 4.92$.

Sedimentation rate was unchanged in the presence of *P*-Ado-*PS* (0.15 mM) or oestrone (9 μM), as indicated by the parallel lines obtained in the presence and

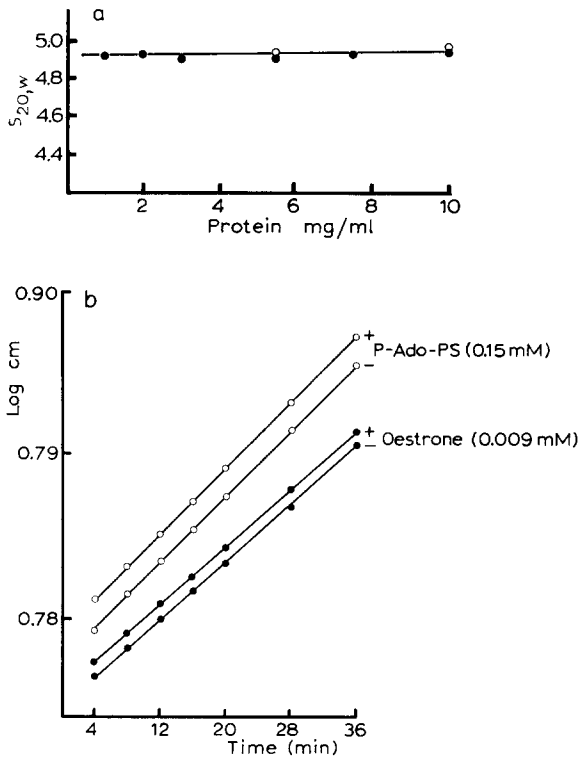

Fig. 2. a. Effect of protein concentration on the sedimentation coefficient of oestrogen sulphotransferase. Samples of enzyme in 0.1 M Tris–HCl, pH 8.2 (●) or 0.1 M Tris–HCl, pH 8.2, containing 0.1 M KCl (○). All experiments were performed at 20 °C and 60 000 rev./min. b. Effect of oestrone and *P*-Ado-*PS* on the sedimentation of oestrogen sulphotransferase. Enzyme (8 mg/ml) was studied in 0.1 M Tris–HCl, pH 8.1. Oestrone (9 μM) was added from a propyleneglycol stock solution so that the propyleneglycol was 1/30 of final volume. The *P*-Ado-*PS* concentration was 0.15 mM. Reference solutions contained the same components minus the substrate. Sample and reference solutions were analysed simultaneously at 20 °C and 60 000 rev./min. Single sector cells were used, one of which contained an upper wedged window and the other conventional windows. The ordinate represents the log of the distance of the boundary (in cm) from the axis of rotation.

absence of these substrates (Fig. 2b). The decrease in sedimentation rate in the oestrogen experiment (Fig. 2b) would appear to be due to the presence of propyleneglycol. Assuming the enzyme was spherical, the $S^2_{20,w}$ value of 4.92 would indicate a molecular weight of 75 000.

Calculation of the partial specific volume from the amino acid composition gave a value of 0.718 g/cm^3. Employing this value, the molecular weight determined by the meniscus depletion method, was 74 800. The results of sedimentation equilibrium studies undertaken to determine the influence of the substrates on the molecular weight, are presented in Fig. 3. In the presence of *P*-Ado-*PS* (0.15 mM), or oestrone (9 μM), the enzyme exists as a single molecular-weight species as shown by the straight line obtained in the log $(Y - Y_0)$ versus r^2 plot. Calculation of the molecular weight of the protein species present from the slope of this line gave a value of 75 000 in each case. A subsequent study of the influence of oestrone plus *P*-Ado-*P*,

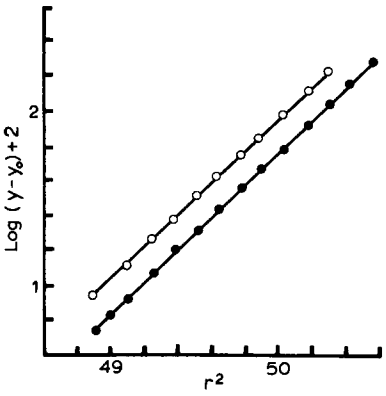

Fig. 3. Sedimentation equilibrium studies on oestrogen sulphotransferase in the presence of substrate. Enzyme (0.5 mg/ml) was present in 0.1 M Tris–HCl, pH 8.2, and added 9 μM oestrone (●) (see Fig. 2b), or 0.15 mM *P*-Ado-*PS* (○). Sedimentation equilibrium (meniscus depletion method) was employed at 20 °C and 21 650 rev./min. The log of the concentration of protein (Yphantis [40]) was plotted against the square of the distance (cm) from the centre of rotation.

once again showed the presence of a single protein species of the same molecular weight. The molecular weight was also determined in the presence of guanidine hydrochloride and also at high pH. A sample of the enzyme in 6 M guanidine hydrochloride was studied by the sedimentation equilibrium, meniscus-depletion method. By use of the formula of Ullman et al. [19] which allows for correction to be made for changes in the partial specific volume of the protein in 6 M guanidine hydrochloride, the molecular weight was calculated from the slope of the line to be 74 500. Sedimentation equilibrium studies in 0.1 M sodium phosphate, pH 12.0, similarly failed to show the presence of subunits; a molecular weight of 73 800 being obtained.

Sucrose-density gradient studies showed that the rate of sedimentation of oestrogen sulphotransferase, as determined by enzyme activity, was independent of pH. The molecular weight determined at pH values of 5.8, 7.0 and 9.0 by the method of Martin and Ames [20], and employing yeast alcohol dehydrogenase as reference protein, was 74 000, 74 800 and 76 000, respectively. Only a single peak of enzyme activity was present in each instance.

Amino acid analysis and tryptic digestion

The amino acid analysis of the reduced S-carboxymethylated protein is shown in Table I. The number of amino acid residues is calculated on the basis of a molecular weight of 74 000. A sample of reduced S-carboxymethylated enzyme was digested with trypsin and a peptide map made by the usual procedure. In excess of 70 ninhydrin spots were observed, which approached the theoretical number based on the amino acid analysis, and furthermore, indicated that the enzyme did not contain a number of identical subunits.

Effect of thiols

The wave-like nature of the kinetic curves obtained with varying oestrogen, and the activation of the enzyme by thiols such as cysteine, have been previously reported [7]. Other thiols have now been investigated. Preincubation of the enzyme

with the thiols shown in Fig. 4 prior to assay, resulted in activation, a higher degree being achieved at low oestrogen concentration. Dithiothreitol however inactivated the enzyme when concentrations of oestrone in excess of 5 μM were used in the assays (Fig. 4). At the same time the kinetic curve approached that of a rectangular hyperbole. The effect of varying dithiothreitol concentration at two different levels of

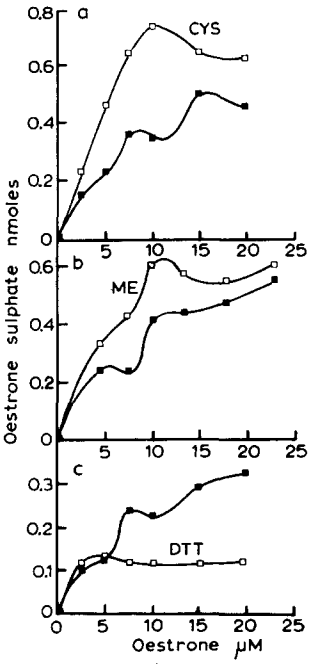

Fig. 4. Effect of thiol on kinetics. Enzyme was preincubated at 37 °C for 10 min in the presence of 10 mM concentrations of the following thiols: (a) cysteine (CYS), (b) β-mercaptoethanol (ME), and (c) dithiothreitol (DTT). Controls were preincubated for 10 min without addition of thiol. Standard enzyme assay conditions used (see Methods) with 50 μg of enzyme and 30 min incubation time. Thiol was maintained at 10 mM in the assay mixture.

oestrogen is shown in Fig. 5. At concentrations of dithiothreitol around 1 mM, the enzyme is activated when assayed at both low (Fig. 5a) and high (Fig. 5b) oestrogen levels. However as the concentration of dithiothreitol is raised, the extent of the activation decreases and reverts to an inhibition as the concentration is raised further. This latter effect was more pronounced, and occurred at lower dithiothreitol concentrations, when high oestrogen levels were used in the assay. A very similar activation–inhibition response to that shown in Fig. 5b occurred when reduced glutathione was used in place of dithiothreitol.

Effect of p-hydroxymercuribenzoate

Initial experiments revealed that the enzyme was inhibited by 5 mM *p*-hydroxy mercuribenzoate when assayed over a range of oestrogen concentrations from 2–60 μM. However inhibition occurred only at oestrogen concentrations less than 12 μM when 0.1 mM *p*-hydroxymercuribenzoate was employed. At a *p*-hydroxymercuri-

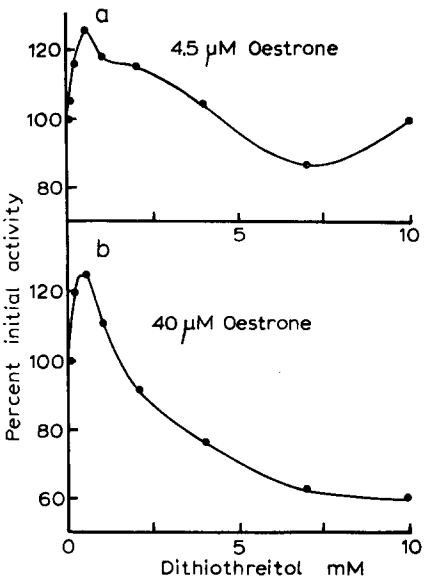

Fig. 5. Effect of dithiothreitol on enzyme activity. Standard assay conditions except Mg^{2+} was not added. Oestrogen levels were as shown and 10 μg of enzyme employed.

benzoate concentration of 0.5 mM, inhibition occurred over the complete oestrogen range. These studies were extended in an experiment in which the enzyme was pre-incubated with 0.4 mM *p*-hydroxymercuribenzoate for various time intervals, then assayed at two different concentrations of oestrogen. A much greater percentage loss in activity occurred when the enzyme was assayed at a low oestrogen concentration (Fig. 6).

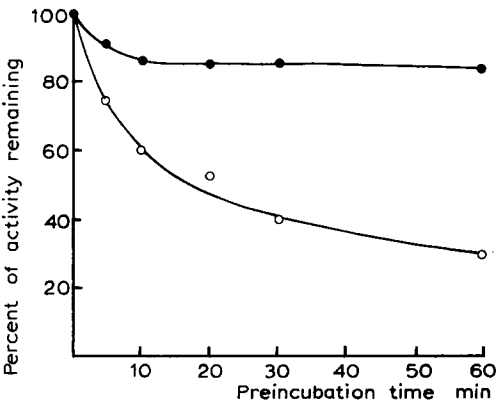

Fig. 6. Effect of time of incubation, in the presence of *p*-hydroxymercuribenzoate, on enzyme activity assayed at two levels of oestrogen. Enzyme was incubated at room temperature in 0.1 M Tris–HCl, pH 8.1, containing 0.4 mM *p*-hydroxymercuribenzoate. Aliquots (30 μg enzyme) were removed at time intervals and activity determined using 6 μM oestrone (○) and 60 μM oestrone (●) in the assays. Bovine serum albumin (250 μg) was added to each assay to remove excess *p*-hydroxymercuribenzoate and an incubation time of 10 min was employed.

Studies on the chemical reactivity of sulphydryl and disulphide groups

Under the conditions employed by the method of Boyer [12], sulphydryl groups could not be titrated in the native enzyme with *p*-hydroxymercuribenzoate. Titration in the presence of sodium dodecylsulphate however resulted in reaction of 1.03 SH groups/mole enzyme. Using Ellman's reagent [11], the native enzyme reacted to yield 0.18 SH group/mole enzyme, whilst in the presence of sodium dodecylsulphate this was increased to 0.90 SH group/mole enzyme. Thus the presence of a 'masked' SH group was indicated.

In order to investigate whether the wave-like kinetic curves may perhaps have been related to substrate-induced conformational changes, the reactivity of the SH group was examined in the presence of substrates. Results are shown in Table I. *P*-Ado-*PS*, in the presence of equimolar amounts of $MgCl_2$, had no effect on the reactivity of the SH group as determined by Ellman's reagent. Similarly ATP was observed to have no effect at the concentrations used. Oestriol would appear to block the titration of the SH group at high concentrations. NaCl at concentrations of 0.15, 0.5 and 1.0 M had a slight but significant effect on the titration; values of 0.28, 0.30 and 0.30 SH group/mole protein being recorded with Ellman's reagent. Addition of NaCl also influenced the rate at which the reaction progressed; the rate increasing with increasing NaCl concentrations.

TABLE I

TITRATION OF SH GROUPS IN OESTROGEN SULPHOTRANSFERASE

Titration was carried out by the Ellman method [11]. The mixture contained enzyme (13.3 nmoles); 0.05 M Tris–HCl, pH 8.2 and additive in the concentrations stated, in a final volume of 1.1 ml. Oestriol was added in 0.1 ml of propyleneglycol. In those titrations containing nucleotide, an equimolar concentration of $MgCl_2$ was also added.

Concn of additive (μM)				Number of SH groups/mole		
ATP	*P*-Ado-*PS*	Oestriol	In presence of:	ATP	*P*-Ado-*PS*	Oestriol
0	3.5	0		0.17	0.16	0.19
5	7	5		0.16	0.17	0.18
10	10	10		0.16	0.17	0.17
20	14	20		0.17	0.16	0.18
100	21	100		0.16	0.17	0.09
	35				0.18	
	50				0.16	

The amino acid analysis (Table II) showed that a total of 23 half-cystines were present. This value was also found in each of the individual isoenzymes analysed. Results would suggest that only a single SH group is present, although the possible presence of other, more deeply buried SH groups, cannot be discounted. Some attempt to titrate disulphide bonds was made using the method of Zahler et al. [13]. In the method, dithiothreitol is used as reductant, and is itself complexed with arsenite at the end of the reaction; liberated SH groups being titrated with Ellman's reagent. However, as pointed out by the authors, this method of titrating disulphides may not be readily applicable to proteins due to the possibility of particular protein SH groups complexing with arsenite after their formation by addition of dithiothreitol. Never-

TABLE II

AMINO ACID COMPOSITION OF OESTROGEN SULPHOTRANSFERASE

Residues/mole in excess of 30 have been rounded off to the nearest whole number. "Intact" enzyme refers to a preparation containing four isoenzymes isolated from DEAE-cellulose columns and was reduced and alkylated with iodoacetic acid prior to hydrolysis. The isoenzymes were isolated from acrylamide slabs after electrophoresis and were hydrolysed without reduction/alkylation.

Amino acid	Residues/mole			
	"Intact" enzyme	Isoenzyme 2	Isoenzyme 3	Isoenzyme 4
Lysine	59	62	59	52
Histidine	15.8	16.8	14.2	15.3
Arginine	23.6	22.9	22.3	23.6
Asparagine	80	87	85	78
Threonine	38	35	35	35
Serine	45	50	47	49
Glutamic acid	67	67	66	68
Proline	29.6	27.4	29.2	30.0
Glycine	51	57	65	82
Alanine	49	56	54	54
Half cystine	21.8**	23.2	24.4	23.0
Valine	40	39	38	38
Methionine	5.9*	9.2	9.4	7.6
Isoleucine	24.7	20.7	19.2	21.0
Leucine	53	50	51	49
Tyrosine	20.7	21.6	22.0	21.0
Phenylalanine	27.6	28.2	28.4	28.4

* Differences in methionine levels between intact enzyme and isoenzymes could be due to non-specific alkylation of these residues by the iodoacetic acid.

** As *S*-carboxymethylcysteine.

theless, the method was tried in an attempt to assess the relative reactivity of disulphide bonds in the presence and absence of detergents. With these limitations in mind, a total of 2.7 SH groups/mole enzyme were titrated using the native enzyme which would equate with one disulphide bond (Table III). Addition of sodium dodecyl-

TABLE III

ATTEMPTED TITRATION OF DISULPHIDES IN THE PRESENCE AND ABSENCE OF DETERGENTS

The method of Zahler and Cleland [13] was employed (see text). Enzyme, 15 mM Tris–HCl, pH 9.0, dithiothreitol (0.75 mM) and denaturing agent, where indicated, were placed in a quartz cell at 20 °C for 1 h. A blank, minus protein, was also prepared. The volume was then adjusted to 2 ml by addition of the following: arsenite (4 mM), 0.2 M Tris–HCl, pH 8.2, and denaturing agent as indicated. After 5 min, 0.02 ml of 10 mM 5,5′-dithiobis(2-nitro benzoic acid) was added and the change in absorbance at 412 nm recorded against time.

Addition	SH groups/mole enzyme
Nil	2.7
Dodecylsulphate, 0.3%	2.2
Urea (6 M)	4.6
Guanidine HCl (4 M)	7.0

sulphate did not greatly alter this result, but in the presence of 6 M urea, or 4 M guanidine hydrochloride, the equivalent of one and two additional disulphide bonds, respectively, were found capable of reacting with the Ellman's reagent under the experimental conditions, i.e. in the presence of arsenite (Table III).

Kinetic studies

Oestrogen varied at constant P-Ado-PS. The complexity of the kinetics obtained by varying oestrogen at constant *P*-Ado-*PS* is seen in Fig. 4. Peaks and troughs were consistently obtained and were accentuated by preincubation of the enzyme at 37 °C. Inclusion of 0.13 mM *P*-Ado-*PS* during this preincubation period did not smooth out the curves or change the positions of the peaks and troughs. Since the pure enzyme, as isolated, contains four individual isoenzymes which travel very close to one another on gel electrophoresis [7], the complexity of the velocity–substrate curves could be due to different kinetic parameters possessed by these isoenzymes. It was thus essential to separate them and determine whether individually they exhibit Michaelis–Menten kinetics. This had now been achieved on a small scale by repeated chromatography on DEAE-cellulose columns. Wave-like kinetic curves were obtained with each of the individual isoenzymes when oestrogen was the variable substrate (Fig. 7). Thus the presence of isoenzymes per se is not the cause of the complex kinetics.

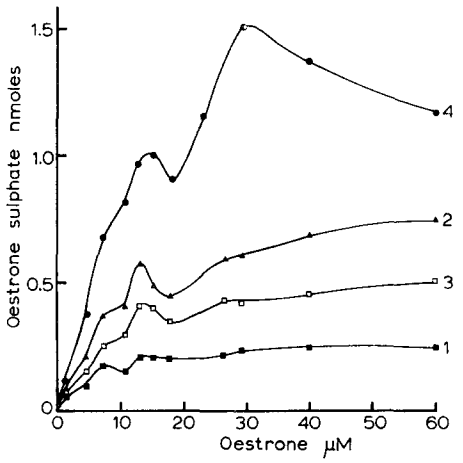

Fig. 7. Kinetics of individual isoenzymes with varying oestrone. Individual isoenzymes were isolated by column chromatography: isoenzymes 1, 2 and 3 on DEAE-cellulose and isoenzyme 4 on DEAE-Sephadex (see Methods). Data on isoenzymes 1–3 were collected from the one experiment using 10 μg protein per incubation in each case. Data on isoenzyme 4 was taken from a separate experiment using 17 μg enzyme per incubation but was corrected to compare with the other isoenzymes within the one figure. Standard enzyme assay procedure was employed but the $MgCl_2$ concentration was 20 mM in place of 15 mM and the incubation time was 15 min.

It should be mentioned at this point that due to this complexity in the velocity–substrate curves it was necessary, when examining the effects of various substances on enzyme activity, to employ a range of substrate, i.e. oestrogen, concentrations. Most of these studies were not done under strictly rigorous kinetic conditions. For

example, product formation exceeded 50% of the initial oestrogen concentration in some cases when low oestrogen levels were used. To a certain extent this was unavoidable since the method of assay measured product formed, and it was necessary to accumulate sufficient counts of oestrogen [^{35}S]sulphate for accurate measurement. However, very similar wave-like kinetic curves were obtained when experiments were performed over shorter incubation periods with *P*-Ado-*P*^{35}*S* of very high specific activity in which conversion of oestrone to oestrone [^{35}S]sulphate was limited to a maximum of 6%.

Over the very limited oestrogen range of 0–8 μM, the kinetics more closely resembled a normal rectangular hyperbola and the K_m for oestrone, calculated from the double reciprocal plot, was 15 μM* and apparently independent of *P*-Ado-*PS* concentration (Fig. 8).

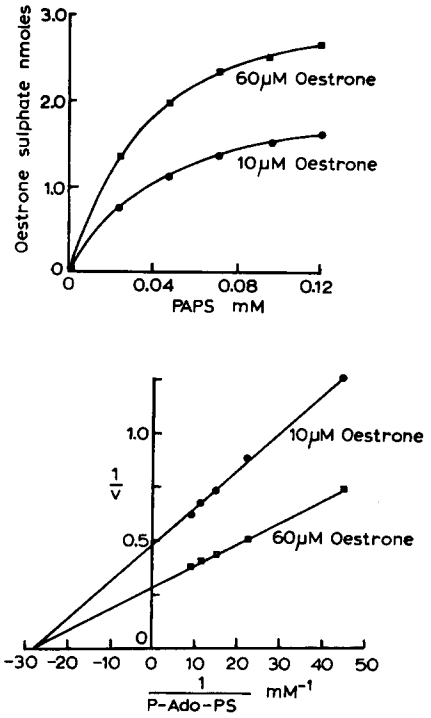

Fig. 8. Kinetics obtained by varying oestrogen over a low concentration range at two different *P*-Ado-*PS* (PAPS) levels. The data is plotted in double reciprocal form in the lower part of the figure.

P-Ado-PS varied at constant oestrogen. In marked contrast to the type of curves shown in Figs 4 and 7, the curves obtained when *P*-Ado-*PS* was varied to near-saturating levels, at different, but constant levels of oestrogen, obeyed Michaelis–Menten kinetics. The double reciprocal plots were linear and the K_m for *P*-Ado-*PS* was 37 μM (Fig. 9).

* An error occurs in Fig. 4 of ref. 18. The reciprocal values of the oestrogen concentrations are ncorrect and recalculation of the K_m for oestrone from this figure also gives a value of 15 μM.

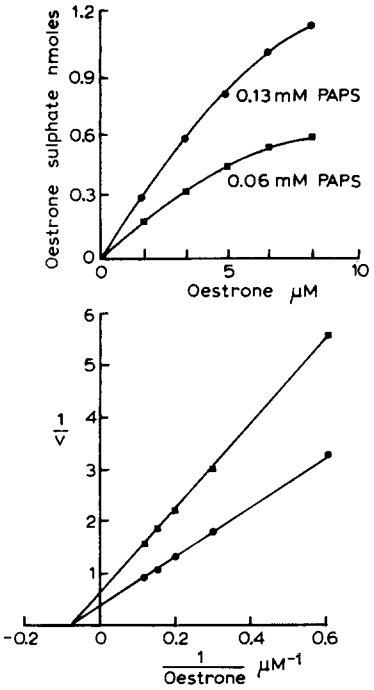

Fig. 9. Kinetics with *P*-Ado-*PS* (PAPS) as variable substrate. Normal assay procedures except that $MgCl_2$ was omitted. The data is plotted in double reciprocal form in the lower figure.

Effect of adenine nucleotides. ADP was a non-competitive inhibitor of *P*-Ado-*PS*; the K_i being 2.8 mM (Fig. 10). This confirms the results reported previously using partially purified enzyme [6]. Inhibition by ATP however was of the mixed type [21] as can be seen in Fig. 10. On plotting $1/v$ versus ATP at two fixed concentrations of *P*-Ado-*PS*, the results shown in Fig. 11 were obtained. Since the inhibition did not increase indefinitely with increasing inhibitor concentration at the low *P*-Ado-*PS* level, a fully competitive inhibition can be ruled out. Furthermore the failure to obtain a straight line relationship would place this type of inhibition in the partially-competitive class [22]. Inhibition by *P*-Ado-*P* was competitive; the K_i value being 0.056 mM (Fig. 12). This suggested that binding to the enzyme involved the 3'-phosphate group and was confirmed by the competitive inhibition exhibited by 3'-AMP (Fig. 13). The K_i value in this case was 1.9 mM.

Isolation of isoenzymes

Although disc electrophoresis on acrylamide gels revealed the existence of four individual components responsible for enzyme activity [7], these bands ran very close together and could not be adequately resolved using preparative gel-electrophoretic methods. Chromatography on ion-exchange columns under a variety of conditions also failed to completely separate the isoenzymes and necessitated repeated chromatography of partially resolved fractions. Samples of isoenzymes 1–4 (numbered in order of increasing electrophoretic mobility as anions) were initially obtained by

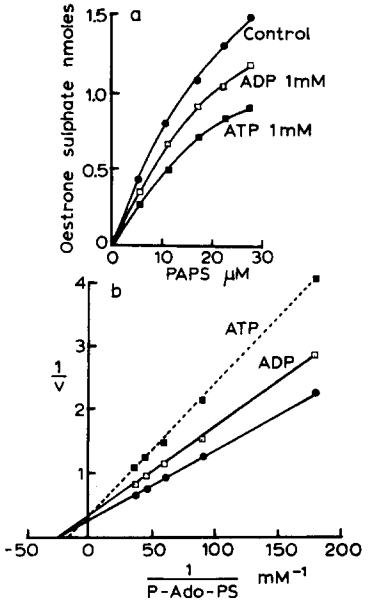

Fig. 10. Effect of adenine nucleotides on kinetics with *P*-Ado-*PS* (PAPS) as variable substrate. $MgCl_2$ was omitted from the assays. The data is plotted in double reciprocal form in the lower figure.

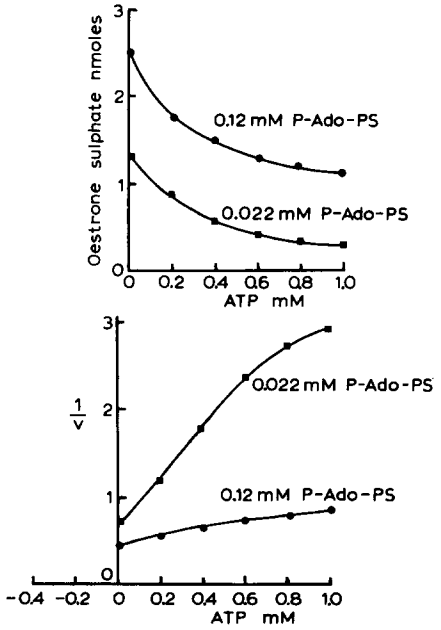

Fig. 11. Effect of varying ATP at two different concentrations of *P*-Ado-*PS*. $MgCl_2$ was omitted from the assays. In the lower figure the reciprocal of the initial velocity is plotted against ATP concentration.

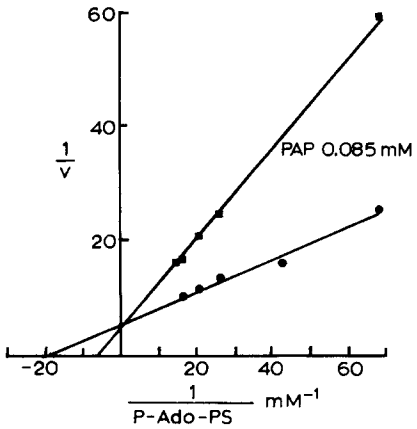

Fig. 12. Competitive inhibition by *P*-Ado-*P* (PAP). Normal assay procedure was used but 17β-estradiol(30 μM) was used in place of oestrone.

elution from acrylamide slabs. When re-run under similar electrophoretic conditions, they behaved as individual species —no reequilibration occurring.

Resolution of isoenzymes on ion-exchange columns. Recoveries of isoenzymes from acrylamide-gel slabs were poor and enzyme activity was low. Ion-exchange procedures were then used. Enzyme was firstly partially purified on a CM-Sephadex C50 column; a procedure which adsorbed some protein but did not retain the enzyme. The latter was then applied to a DEAE-Sephadex (A-50) column and a linear NaCl

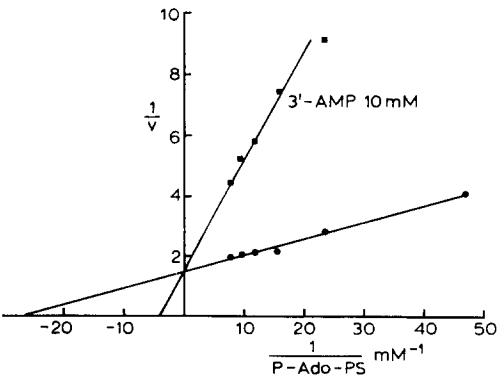

Fig. 13. Competitive inhibition by 3′-AMP.

gradient applied. Three peaks of enzyme activity were found (Fig. 14). Aliquots of fractions 34, 47 and 58, i.e. fractions corresponding to the three peaks of activity, were examined by sucrose gradient ultracentrifugation (Fig. 15). All three samples showed a zone of enzyme activity just in front of hemoglobin. Fraction 34, the peak fraction from the first area of enzyme activity eluted from the DEAE-Sephadex, also showed an additional zone towards the bottom of the tube (Fig. 15). Analysis of the proteins present in the three peaks of enzyme activity of Fig. 14, were studied by disc

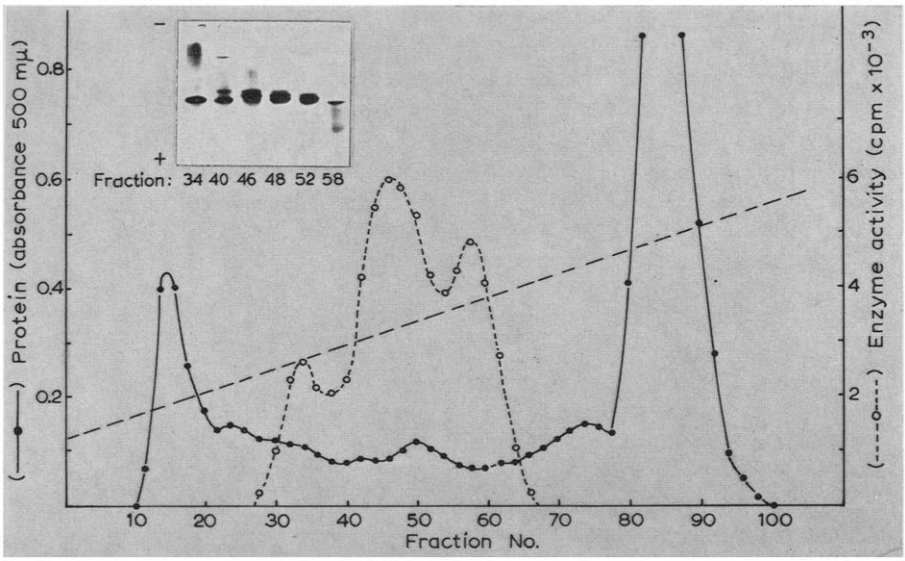

Fig. 14. Partial resolution of isoenzymes by chromatography on DEAE-Sephadex. Enzyme was prepared to the $(NH_4)_2SO_4$ fractionation stage and then passed through a column of CM-Sephadex (C-50) equilibrated with 0.05 M phosphate, pH 6.2. Oestrogen sulphotransferase was not adsorbed to this column but hemoglobin and other proteins were removed. The enzyme fraction was dialysed against 0.025 M Tris–HCl, pH 8.0, containing 0.06 M NaCl. It was then applied to a 4.4 cm × 30 cm DEAE-Sephadex (A-50) column equilibrated with the same buffer. A linear 0.06–0.3 M NaCl gradient (1 l) was passed through the column at a flow rate of 30 ml/h and 15-ml fractions collected.

electrophoresis. Fraction 34 contained isoenzyme 3, together with a slower-running band, which very likely represented the high molecular weight species detected by sucrose-gradient centrifugation. Partial resolution of the isoenzymes then occurred, the isoenzymes appearing in order of increasing negative charge. Fraction 40 contained isoenzymes 1 and 3; fraction 46, 1, 2 and 3; fraction 48, 1, 2 and 3; fraction 52, 2, 3 and 4 and fraction 58 isoenzyme 4 only (Fig. 14). It would appear that the first zone of enzyme to be eluted contained an associated species of isoenzyme 3, which was capable of dissociation as shown by the gel pattern and by ultracentrifugation on a sucrose gradient.

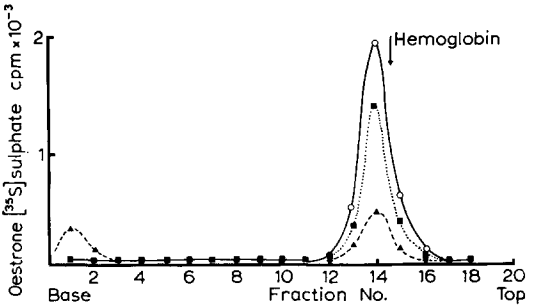

Fig. 15. Sucrose-density gradient ultracentrifugation studies on the three peak fractions of enzyme activity shown in Fig. 2. Fraction 37 (▲), fraction 47 (○) and fraction 58 (■).

Partial resolution of the isoenzymes also occurred on columns of DEAE-cellulose. Enzyme was again eluted in more than one zone (Fig. 16). A section of the elution profile is enlarged to show the isoenzyme patterns revealed by disc electrophoresis. The specific activity was constant as isoenzyme 1 was eluted (fractions 10–13), then rose as isoenzyme 2 appeared (fractions 14–16), became constant again as isoenzyme 3 appeared (fractions 17–20), then rose again as isoenzyme 4 was eluted (fractions 21–26). It was obvious from this study that the isoenzymes varied consider-

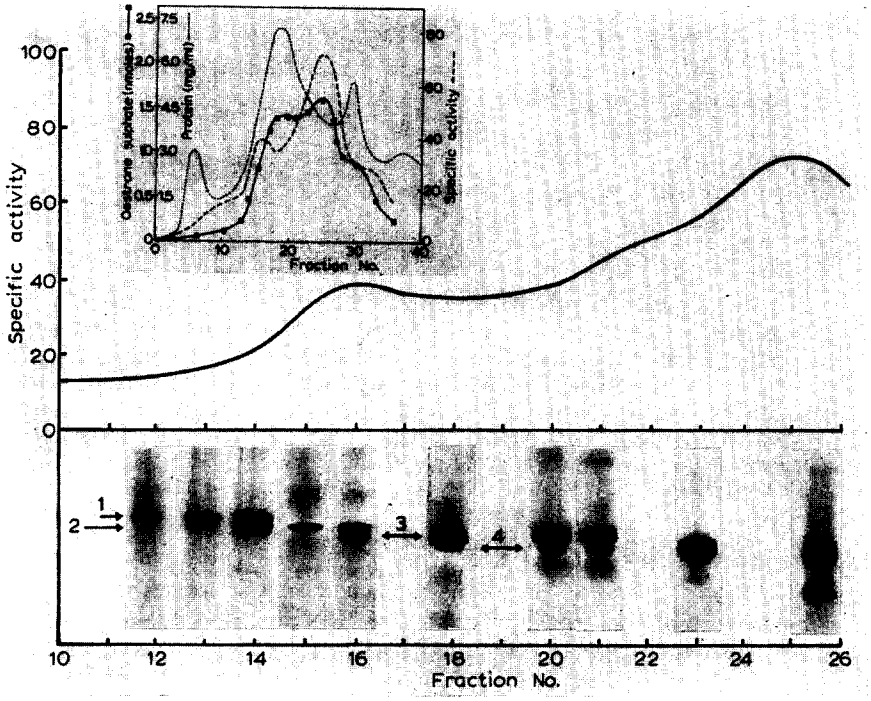

Fig. 16. Partial resolution of isoenzymes by chromatography on DEAE-cellulose. Details of elution conditions are given in Methods and the elution profile is shown in the insert. Acrylamide-gel patterns of selected fractions from tube 10 to tube 26 are shown. Note the change in specific activity with the elution of the different isoenzymes (indicated by arrows).

ably in their specific activities and explained why multi-peaks of enzyme activity were observed on elution from ion-exchange columns (Figs 14 and 16). By re-running those fractions, which were predominately of one isoenzyme type only, on smaller columns of DEAE-cellulose, mg quantities of isoenzymes 1–3, of at least 95% purity, were obtained. It was not possible to obtain isoenzyme 4 by this method. However a pure sample of this isoenzyme was obtained by chromatography on DEAE-Sephadex (Fig. 14). Specific activities for isoenzymes 1 to 4, at 50 μM oestrone, were 25, 75, 50 and 117, respectively.

Possible subunit structure

Many isoenzymes represent hybrid molecules composed of combinations of different subunits. The possible occurrence of subunits in oestrogen sulphotransferase

was further examined by (i) electrophoresis in acrylamide gels containing either urea or sodium dodecylsulphate and (ii) by maleylation followed by gel filtration.

Fig. 17 shows the effects of increasing concentrations of urea on both enzyme activity and gel electrophoretic patterns. Urea concentrations greater than 2 M caused a loss of enzymic activity, apparently by formation of aggregates. No evidence of dissociation to lower molecular weight subunits was discernible. Addition of urea could conceivably cause intermolecular disulphide bond formation initiated by the single SH group present in the enzyme. However, when the enzyme was reduced and S-carboxymethylated prior to electrophoresis in 8 M urea gels, then protein bands still appeared in the slowly migrating aggregate region. Intermolecular disulphide bonding would then not be the exclusive reason for aggregation.

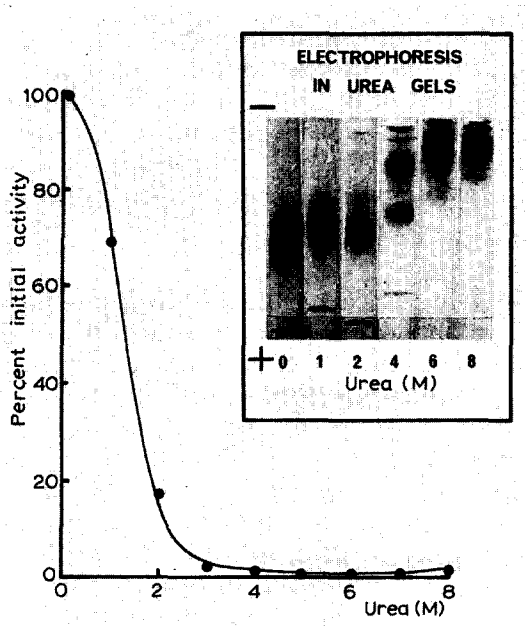

Fig. 17. Effect of urea on acrylamide-gel pattern and activity of the enzyme. Acrylamide gels incorporating 0–8 M urea were prepared, and enzyme (100 μg), in the same concentration of urea, was loaded onto the gel. Enzyme was assayed by the standard procedure in the presence of urea at the concentrations indicated.

The S-carboxymethylated enzyme was reacted with maleic anhydride under dissociative conditions [15]. On gel filtration on a column of Sephadex G-75, no evidence for subunits was found; the maleylated enzyme being eluted slightly before the untreated enzyme.

Treatment with sodium dodecylsulphate and thiol followed by electrophoresis in acrylamide gels containing the detergent [17], again failed to show the presence of subunits. The enzyme as normally isolated, and the individual isoenzymes 1–4, when examined separately by this technique, were found to have molecular weights near 74 000 by comparison with reference proteins.

Electrophoresis in gels of varying strengths

The method of Hedrick and Smith [23] was used to determine whether the isoenzymes belonged to the "charge isomer" (proteins having the same size but different nett charges) or "size isomer" (proteins having different sizes but the same charge/mass ratios) classifications. These classifications are distinguished by parallel lines or non-parallel lines, respectively, when 100 (log $R_m \times 100$) is plotted against gel concentration. Parallel lines were obtained in this instance thus demonstrating that the isoenzymes had the same, or nearly the same, molecular weights but differed in charge.

Is the binding of small molecules responsible for the gel patterns?

The possibility that charge differences may have arisen by binding of small charged molecules was investigated. On analysis the enzyme was found not to contain carbohydrate or phosphate groups. If a non-covalently bound molecule was responsible for the charge difference, then its addition, in excess, to the enzyme might be expected to alter the characteristic electrophoretic pattern. The enzyme was therefore incubated with various divalent metal ions, adenine nucleotides, the enzyme's substrates and products, protein phosphate phosphorylase and lipase, and in addition, was extracted with ether. None of these treatments altered the electrophoretic pattern.

Possibility of artefact formation by proteases

When an enzyme exhibits multiple forms which cannot be definitely attributed to genetic phenomenon, the question must always be asked as to whether they may be artefacts. Such artefacts sometimes arise by cleavage of peptide bonds by proteases released during the tissue disruption. Examples are yeast hexokinase [24] and chicken aldolase [25]. Since many of the proteases contain serine at the active site, which can be effectively inhibited by diisopropylfluorophosphate, the adrenal enzyme was isolated on a small scale in the presence and absence of this inhibitor at 1 mM concentration. Resultant enzyme activities were identical and there was no alteration in the characteristic 4-banded electrophoretic patterns. When a sample of the enzyme as normally isolated, and a sample of isoenzyme 1, were incubated with 1:1000 and 1:10 000 concentrations of trypsin, again no changes in the patterns were observed.

Treatment with urea

Conformational isoenzymes can arise from polypeptides which have the ability to sponteneously assume more than one stable conformation [26, 27]. Variations in electrophoretic mobility may then arise as a result of differences in exposed charge among the conformers. A convenient way to study conformational variation is by reversible unfolding of the protein carried out initially in the presence of thiol and a denaturing agent such as urea. An example of this approach is the study of different forms of chicken heart mitochondrial malate dehydrogenase [28]. Oestrogen sulphotransferase containing the normal 4 isoenzyme components, and a separate sample of isoenzyme 1, were denatured in 8 M urea, containing 0.1 M β-mercaptoethanol. The solution was then dialysed and reoxidation allowed to proceed by slow stirring in air. No conversion of isoenzyme 1 to any other isoenzyme was observed, nor was there any change in the isoenzyme pattern of the normal enzyme. However

this treatment resulted in complete loss of enzyme activity and over half of the protein was rendered insoluble.

Amino acid analysis of isoenzymes

The amino acid analysis of the isoenzymes isolated from acrylamide gel slabs is given in Table II. Insufficient isoenzyme 1 was available for reliable analyses. Residues per mole of protein were calculated on the basis of a molecular weight of 74 000. Although the amino acid analyses are very similar, there are significant differences in the number of Lys, His, Asp and Met residues. Since glycine was used in the electrophoresis buffer during isoenzyme separation, the Gly values in Table II are probably not meaningful. The ratio of Lys to acidic amino acids decreased progressively for isoenzymes 1–4, which correlated with the observed electrophoretic mobility of the isoenzymes. That differences in amino acid compositions may be responsible for electrophoretic variance is then consistent with the analytical data. However the proportion of acidic amino acids present as amides is not known.

Isoenzyme compositions of individual adrenal glands

Isoenzyme patterns in the enzyme purified to the $(NH_4)_2SO_4$ fractionation stage only, were examined in some 30 individual adrenal glands. The four-banded pattern on the acrylamide gels was apparently present in the majority of cases, as far as could be ascertained amongst the complexity of protein bands revealed after staining. Enzyme extracted from individual glands of a small number of animals was then separately purified to the first DEAE-cellulose chromatography stage. The isoenzyme patterns were then examined by disc electrophoresis and the relative amounts determined by densitometry (Fig. 18). No apparent sex differences were observed. In one case however (gland 6), isoenzyme 4 was missing and was accompanied by a

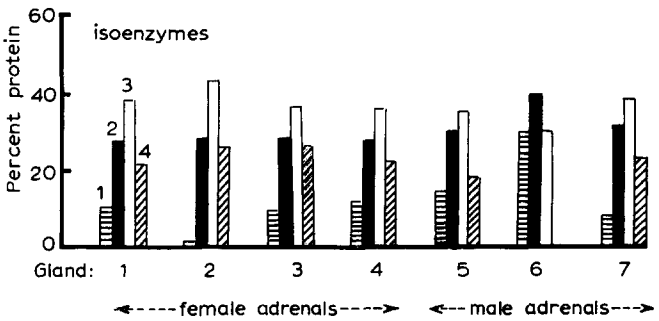

Fig. 18. Quantitation of isoenzyme patterns in enzyme isolated from adrenals of individual animals. Enzyme was purified to the first DEAE-cellulose column purification stage and subjected to electrophoresis on slabs of acrylamide gel. The individual isoenzymes are expressed as a percentage of the total oestrogen sulphotransferase protein present as revealed by staining (see Methods).

change in the relative amounts of other isoenzymes, in particularly isoenzyme 2 now became predominant, in contrast to isoenzyme 3 in the other patterns. An identical situation also occurred amongst the isoenzyme components of three separate batches of enzyme isolated from the placenta of single animals. In other cases, whereby a single placenta was used to prepare enzyme, a four-band pattern was obtained [29].

DISCUSSION

Oestrogen sulphotransferase appears to exist as a single polypeptide of mol. wt 75 000 stabilized by disulphide bonds. At least one "masked" sulphydryl group is present and on this basis up to 10–11 disulphide bonds would occur. Molecular weight determinations by sedimentation velocity, sedimentation equilibrium and sodium dodecylsulphate gel electrophoretic methods, were in good agreement. No evidence was obtained for the presence of subunits by means of sedimentation equilibrium studies in guanidine hydrochloride or in buffers of high pH values, or by molecular weight determinations by gel electrophoresis in sodium dodecylsulphate [9]. All samples of enzyme showed polymorphism by electrophoresis on acrylamide gels. Four such variants exist termed isoenzymes 1–4. From the parallel lines obtained in gels of increasing strength [23], the isoenzymes would be charge variants and analytical evidence indicates that differences in amino acid compositions are responsible (Table II). The likelihood that these isoenzymes represent genetic variants will be discussed later.

In previous studies two forms of the enzyme, termed A and B, were isolated using DEAE-cellulose columns and it was suggested that these may have been related to differing states of association [6]. However it is now apparent that the partial resolution of the isoenzymes, which occurs on chromatography on DEAE-Sephadex or DEAE-cellulose columns, causes more than one peak of activity to appear. This is due to the great difference in specific activity of the isoenzymes; isoenzyme 4 having the highest specific activity. It was also previously thought that these A and B forms had different kinetic properties; one exhibiting normal Michaelis–Menten kinetics and the other showing peaks and troughs on varying oestrogen at constant *P*-Ado-*PS* [7]. This is now known to be due to insufficient points being determined in these earlier studies —all enzyme preparations obtained from adrenal tissue and bovine placenta [29] exhibit the characteristic undulating curves when oestrogen is varied at fixed *P*-Ado-*PS* concentrations.

Very similar complex velocity–substrate curves were obtained using high-speed supernatants, or partially purified fractions, derived from human adrenal tissue and dehydroepiandrosterone as variable substrate [30]. In this instance an association–dissociation system, relating to steroid sulphotransferase activity, was shown to exist whose equilibrium could be altered by substrate, cysteine, Mg^{2+}, etc. It was thus possible that an association–dissociation system, the components of which possessed different enzyme activities and whose equilibrium position was altered by the ratio of substrates, could account for the undulating kinetics exhibited both by the human adrenal system and the purified bovine oestrogen sulphotransferase. The presence of a single symmetrical boundary, as revealed in sedimentation velocity studies of oestrogen sulphotransferase, would apparently eliminate the existence of an associating system based on the theory of Gilbert [31]. This theory predicts a single unsymmetrical boundary if $n = 2$, but two boundaries if $n > 2$, in the equilibrium $A \rightleftharpoons nB$. Rao and Kegeles [32], in an extention of the theory of Gilbert, have shown however that in a system in which monomer, dimer and trimer were all present in equilibrium, only a single boundary is formed. In such a system the molecular weight of the sedimenting species would be the mean molecular weight of the protein species present. A plot of $s_{20,w}$ against protein concentration (Fig. 2a), however, indicated that the

value was independent of protein concentration, at least over the range studied, and if an associating system was present, an increase in the mean molecular weight would have been expected. Under strongly dissociative conditions, such as those used in the determination of molecular weight by gel electrophoresis in the presence of sodium dodecylsulphate the molecular weight was unchanged. Again, the molecular weight, as determined in guanidine hydrochloride or in buffers or high pH, was not significantly altered. Such results would indicate the absence of association in the normal sedimentation runs. The presence of oestrone or *P*-Ado-*PS* failed to alter the sedimentation rate of the enzyme (Fig. 2b). Using sedimentation equilibrium methods, protein concentrations nearer those used in kinetic studies were able to be employed and again addition of either oestrone or *P*-Ado-*PS*, or oestrone plus *P*-Ado-*P*, did not alter the molecular weight (Fig. 3). Measurement of enzyme activity in sucrose gradient centrifugation runs also showed only a single peak of molecular weight near 75 000 and apparently unaltered by pH variation in the range 5.8–9.0. Thus no support for the existence of an association–dissociation system was found, which makes this a most unlikely explanation for the undulating kinetics exhibited by oestrogen sulphotransferase. However in some enzyme preparations, involving the use of DEAE-Sephadex columns, partial resolution of isoenzymes occurred and was accompanied by the appearance of aggregates of isoenzyme 3 (Fig. 14). It would also seem that despite evidence for an association–dissociation system applying to the steroid alcohol sulphotransferase in human adrenal preparations [30], this, in itself, is probably not the primary cause for the very similar undulating kinetics found with this enzyme. An alternative mechanism applicable to both enzymes would more likely explain the phenomenon.

Teipel and Koshland [33] have shown that a combination of positive and negative cooperativity with respect to K_{cat} or K' (intrinsic binding constant) can result in intermediate plateau, or "bumpy" regions, in enzyme kinetic curves, i.e. when the relative magnitudes of the catalytic or binding constants first decrease then increase as the enzyme is saturated. For this theory to be relevant however, the enzyme must possess more than two substrate binding sites. Multiplicity of binding sites almost always implies a protein composed of subunits and it is, in turn, the substrate-induced conformational change in the subunit which provides the basis for Koshland's sequential model to explain positive and negative cooperativity in oligomeric proteins [33]. Enzymes which exhibit bumps in their saturation curves include phosphoenolpyruvic carboxylase, yeast triose phosphate dehydrogenase, threonine deaminase and *Escherichia coli* ADP-glucose pyrophosphorylase [34]. These enzymes are composed of subunits and thus likely to possess the required number of binding sites in keeping with this theory. Available evidence would then argue against this theory providing the explanation for the wave-like saturation curves exhibited by oestrogen sulphotransferase.

Plateau, or perhaps bumpy regions in velocity–substrate curves, could also arise from the presence of a number of distinctive enzyme species with different kinetic parameters. The existence of individual isoenzymes, as components of the enzyme as normally isolated and studied, could then provide the explanation. This possibility was in turn eliminated by the retention of the wave-like features by each individual isoenzyme (Fig. 7). Elimination of the presence of a mixture of isoenzymes, as an explanation for the wave-like kinetics exhibited by the enzyme as normally isolated,

did not remove the possibility that various conformational forms of the polypeptide chain may exist and thus equate with the "number of distinctive enzyme species" referred to above. Evidence for the presence of these distinctive enzyme species, referred to hence forth as "conformational" forms, will now be presented.

Titration of only 0.2 SH group per mole of enzyme in the native state and the increase to approximately 1.0 SH group per mole in the presence of sodium dodecylsulphate, suggests that the enzyme contains a single "masked" SH group. In this respect the enzyme is similar to bovine serum albumin; the SH group of which is titrated to the extent of 0.6 residue per mole in the native state and is not fully titratable until detergent is added [35]. Jensen [35] has explained this "masking" as being due to a stable but reversible combination with some other group in the protein. The masking observed in oestrogen sulphotransferase need not involve such an interaction but may be due to the SH being buried in the folds of the three-dimensional structure. The amino acid composition of the enzyme bears a striking similarity to bovine serum albumin and it is possible that the two proteins may be genetically related [36]. Current work is proceeding to examine the amino acid sequence around the single SH group in both proteins to test for possible homology. Possession of a SH group in a protein stabilized by disulphide bonds is uncommon and such structures are potentially unstable due to the possibility of disulphide exchange. Schachman [37] has suggested that in the case of serum albumin, disulphide exchange could provide a large number of conformers and thus help to provide the adaptability required of serum albumin in its physiological role as a transport protein.

Addition of cysteine or mercaptoethanol to the enzyme resulted in activation; the degree of activation however depended on the oestrogen concentration employed. A greater degree of activation resulted with low oestrogen levels in the assays (Fig. 4). Dithiothreitol also activated at low oestrogen levels but inhibition occurred at higher oestrogen concentrations; the undulations being smoothed out into something approaching Michaelis–Menten kinetics (Fig. 4). Low concentrations of dithiothreitol caused activation and inhibition was only seen as the concentration was raised (Fig. 5). This inhibition was much more pronounced when the enzyme was assayed at high oestrogen concentrations (Fig. 5b). If we now consider the effects of an SH-blocking agent such as *p*-hydroxymercuribenzoate, we again find an effect —in this case an inhibition— which is dependent upon the concentration of oestrogen used in the assay (Fig. 6). It is suggested that the effects of thiols and the SH-blocking agent can be explained by the presence of enzyme conformers which differ particularly in their affinity for oestrogen. A low K_m species is activated by thiols and possesses a SH group in a more exposed position and is therefore more sensitive to low concentrations of *p*-hydroxymercuribenzoate. Since only one disulphide bond was titrated in the absence of denaturing agents* (Table III), the enzyme could possess a SH and disulphide bond(s) in close proximity and thus allow for intramolecular scrambling to occur by disulphide exchange. Conformers could then exist which have higher K_m values for oestrogen and which have their SH group in a less exposed position than in the low K_m conformer(s). They would therefore be less sensitive to *p*-hydroxymer-

* This result must be interpreted within the limitations set by the method used as mentioned in Results. Additional disulphides were titrated only when detergent was added which does suggest a differential availability.

curibenzoate. Since the enzyme exhibits normal Michaelis–Menten kinetics when *P*-Ado-*PS* is varied at constant oestrogen levels, conformational differences would be primarily confined to areas involved in the binding of oestrogen. This could implicate the SH group at or near the binding site of oestrogen and is consistent with the evidence discussed above. Further support for positioning the SH group in the vicinity of the binding site of estrogen has been obtained using affinity labelling with 4-mercuriestradiol. Binding of this compound to the SH group, as determined by mercaptide formation measured spectrophotometrically, paralleled loss of enzyme activity (Adams, J. B., and Jackson, D., unpublished). The low K_m and high K_m conforms are depicted below.

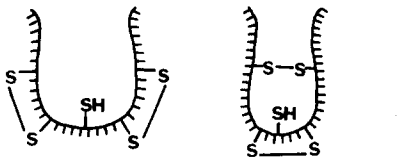

Diagram I.

It is considered that in the scrambling of the disulphides, the SH group would act as an initiator only and would be regenerated in its original position. If we again compare the situation in bovine serum albumin, this protein has been shown to undergo a SH-catalysed structural alteration to produce a new protein species with a different isoelectric point and optical rotation properties [38]. A multiple disulphide exchange was inferred; the SH group playing merely a catalytic role since labelling of the SH group and peptide mapping indicated that the SH group did not shift to a new position [38].

Activation of the enzyme by thiols may be due to some reversal of the scrambling with generation of higher concentrations of the low K_m conformer. However removal of a heavy metal bound to the SH group, or perhaps freeing the SH group from some intramolecular association are possible explanations. Because of the very low redox potential of dithiothreitol (—0.33 V at pH 7.0), the smoothing of the kinetics caused by dithiothreitol (Fig. 4) could result from elimination of higher K_m conformers by reduction of a disulphide bond(s) in a more exposed position in such conformers and thus rendering them enzymically inactive (see Diagram I). Substrate-induced conformational changes could not be detected, as judged by titration of the SH group (Table I). Some further evidence for the proximity of the SH group to the binding site of oestrogen was seen from the reduction in the titre in the presence of high oestriol concentrations, but not in the presence of *P*-Ado-*PS* or ATP (Table I). Fig. 9 shows that the binding of *P*-Ado-*PS* is independent to that of oestrogen. Furthermore, since normal Michaelis–Menten kinetics were obtained, the abnormal behaviour of the enzyme must relate to oestrogen binding. Indeed, preincubation of the enzyme with *P*-Ado-*PS* did not significantly alter the position of peaks and troughs in the velocity versus oestrogen plots. From inhibition studies shown in Figs 10–13, it can be concluded that *P*-Ado-*PS* is bound to the enzyme at the 3′-phosphate group and by comparison of the K_i values of 3′-AMP (1.9 mM) and *P*-Ado-*P* (0.056 mM), it is very likely that the 5′-phosphate group is also involved in

the binding of *P*-Ado-*P* and *P*-Ado-*PS*. ADP cannot occupy the binding site, but ATP, because of the additional phosphoanhydride, can conceivably bridge onto the binding sites normally occupied by 3′- and 5′-phosphate groups. This would be in keeping with the observed non-competitive inhibition by ADP (Fig. 10) and the partially competitive inhibition by ATP (Figs 10 and 11).

The overall interpretation of the data is consistent with the conclusions reached by Barford and Jones [39] on studies with rat liver phenol sulphotransferase. Freshly prepared enzyme sulphurylated *p*-nitrophenol and L-tyrosine methyl ester and displayed normal Michaelis–Menten kinetics; the K_m values being 1.5 μM and 2.9 mM, respectively. After standing at 0 °C for three weeks, the K_m and V for *p*-nitrophenol increased 200-fold and 4-fold respectively. When assayed at intermediate times of standing at 0 °C, kinetic curves containing a bump region were obtained when *p*-nitrophenol was the variable substrate. These changes in kinetic properties were reversible upon addition of thiol. Significantly, the kinetics with *P*-Ado-*PS* as variable substrate were of the Michaelis–Menten type and the K_m for *P*-Ado-*PS* was unaltered during the storage period, indicating an independent variation in the properties of the two substrate-binding sites.

It is difficult to predict whether the various K_m species of oestrogen sulphotransferase actually exist in vivo and have some physiological function. Although steroid sulphotransferases are "soluble" they may in fact be loosely bound to the membrane surface in order to utilise membrane-bound steroid substrates, the resulting steroid sulphate then being released from the membrane surface. Such binding of the enzyme might conceivably stabilise it against the possibility of disulphide-exchange reactions initiated by a SH group.

If we now return to the question of the polymorphism exhibited by the enzyme, the nature of their molecular forms can be considered as belonging to one of the two groups, nongenetic and genetic, as classified by Kaplan [27]. The non-genetic variants are due to (i) artefacts, such as binding of small molecules or the action of proteases, (ii) aggregates, and (iii) conformers in which enzymes have the same molecular weight and amino acid sequences but differences in conformation results in altered electrophoretic mobilities. Genetic variants are due to (i) multiple forms due to separate genes and forming hybrids, e.g. lactate dehydrogenase, creatine kinase, (ii) duplicate enzymes due to different genes but with only slight differences in sequence, and (iii) alleles of the same gene. As far as can be judged, it is considered unlikely that the oestrogen sulphotransferase variants fall into the nongenetic classification. The possibility of protease action, which has been found responsible for multiforms of some enzymes [24, 25], was investigated by use of the serine protease inhibitor diisopropylfluorophosphate, but this reagent when added to tissue homogenates was without effect on the final gel pattern. However, since extensive cleavage of peptide bonds would result in loss of enzyme activity, carboxypeptidases and aminopeptidases would perhaps be more likely to be involved in any artefact formation. No evidence was obtained that differential binding of small ions was responsible or that the variants represented conformers as judged by the failure to observe differences in gel patterns after treatment with urea and thiol. Turning to genetic variants, the possibility of multiple forms resulting from separate genes coding for distinct subunits which can form hybrids, can be discounted since oestrogen sulphotransferase possesses only one polypeptide chain. The isolation of enzyme from individual animals

in which isoenzyme 4 was absent and was accompanied by a predominance of isoenzyme 2, as compared to the normal predominance of isoenzyme 3 when all four components are present (Fig. 18), could perhaps indicate that isoenzymes 2 and 4 are produced by allelic genes. Lack of expression of isoenzyme 4 would then correspond to the homozygosity for the allele producing isoenzyme 2. Isoenzymes 1 and 3 could then possibly correspond to allelic forms of a different genetic locus. Verification of this hypothesis would require the examination of large numbers of animals. The presence of a three-banded pattern was more prevalent in enzyme prepared from placental tissue obtained from individual animals, as compared to enzyme derived from adrenal tissue. It is thus possible that proteases, released from placental tissue on homogenisation, are responsible for the three-banded gel pattern. This could implicate proteases as being the ultimate cause of the polymorphic forms of the enzyme rather than the alternative genetic derivation.

The wave-like features exhibited in the kinetic curves with oestrogen varied, demonstrate that these features are due to an intrinsic property of the polypeptide chain itself. Peaks and troughs were found at very similar oestrogen concentrations for each isoenzyme and it would appear that for the model proposed, each isoenzyme has the ability to adopt conformations with differing affinity for oestrogen.

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