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ENZYMIC SYNTHESIS OF STEROID SULPHATES

III. ISOLATION AND PROPERTIES OF ESTROGEN SULPHOTRANSFERASE OF BOVINE ADRENAL GLANDS

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SUMMARY

1. Estrogen sulphotransferase (3'-phosphoadenylylsulphate:estrone sulphotransferase, EC 2.8.2.4) has been isolated from bovine adrenal glands free of other types of sulphotransferase enzymes.

2. Two forms of the enzyme were isolated by chromatography on DEAE-cellulose and the properties of the more stable form were studied.

3. A pH optimum of 8 was found. The enzyme was activated by cysteine but was only moderately sensitive to SH-blocking agents.

4. Although an absolute requirement for metal ions was not shown, the enzyme was activated by Mg^{2+}, Ca^{2+} and Mn^{2+} but strongly inhibited by Zn^{2+}, Co^{2+} and Ni^{2+}. EDTA at concentrations up to 20 mM did not show inhibition of activity measured in the absence of metal ions.

5. The enzyme was specific for natural estrogens, yielding a monosulphate of the phenolic hydroxyl group. Simple phenols, 2-naphthylamine and 3 β-hydroxysteroids were not sulphated. Stilbestrol and hexestrol, but not dienestrol, were sulphated at very low rates.

6. Kinetic studies revealed that the mechanism was of the sequential type. K_m values for 17 β-estradiol and adenosine-3'-phosphate-5'-phosphosulphate were 14 and 70 μM respectively. The K_m value for the nucleotide was unchanged in the presence of Mg^{2+} suggesting that the metal ion effect involved the $v_{max.}$ rather than the binding of the nucleotide to the enzyme.

INTRODUCTION

Recent publications have demonstrated that steroid sulphates constitute intermediates in steroid metabolism. Thus pregnenolone sulphate and cholesteryl sulphate

Abbreviation: PAPS, adenosine-3'-phosphate-5'-phosphosulphate.

served as *in vivo* precursors of urinary dehydroepiandrosterone sulphate in female subjects with an adrenal adenoma[1,2]. By the use of double labelling experiments, it was shown that the sulphate group remained intact during these conversions[2]. Furthermore *in vivo* studies have emphasised the high efficiency of dehydroepiandrosterone sulphate as a precursor for estrogen synthesis in pregnant women[3,4], and for testosterone and androstenedione synthesis in canine testis[5]. The significance of these observations is enhanced by the demonstration of the secretion of dehydroepiandrosterone sulphate by the human adrenal gland[6,7].

The physiological importance of steroid sulphates naturally focuses attention on the enzymes responsible for steroid sulphation—the steroid sulphotransferases. Attempts at fractionating the sulphokinases of liver preparations were first made by NOSE AND LIPMANN[8] who were able to separate a sulphokinase fraction which sulphated steroid alcohols from one which sulphated estrone and *p*-nitrophenol. More recently, BANERJEE AND ROY[9] have attempted the fractionation of guinea pig liver extracts on columns of Sephadex G-200 and DEAE–Sephadex but only partial resolution was achieved. The presence of such enzymes, capable of sulphating a rather broad spectrum of steroid alcohols and also estrogens, has been demonstrated in normal human adrenal glands[10] (Part I of this series). Due to the difficulty of obtaining sufficient human glands for enzyme fractionation studies, bovine glands were then investigated. The latter exhibited a species difference compared to the human: although high levels of estrogen sulphokinase were present, only very low levels of other steroid sulphokinases were found. The estrogen sulphotransferase (3'-phosphoadenylylsulphate:estrone sulphotransferase, EC 2.8.2.4) has been isolated from this tissue free of other types of sulphokinases and its properties studied.

MATERIALS

Estrogens

The source of the estrogens was as follows: estrone, Light and Co.; 17 β-estradiol, Mann Research Laboratories; estriol and 1,3,5(10)-estratriene-3-hydroxy-17 β-hydroxy-16-one, California Corp. for Biochemical Research; 17 β-estradiol-3-methyl ether and 17 α-estradiol, Sigma Chemical; equilenin, K. and K. Laboratories. Stilbestrol, hexestrol and dienestrol were the highest grades available commercially.

Labelled compounds

[4-^{14}C]estrone, 17 β-[4-^{14}C]estradiol and sodium [^{35}S]sulphate were purchased from the Radiochemical Centre, Amersham.

^{35}S-labelled adenosine-3'-phosphate-5'-phosphosulphate (PAPS)

Radioactive [^{35}S]PAPS of high specific activity was obtained by incubation of rat liver supernatant preparations with ATP, Mg^{2+} and [^{35}S]sulphate by the method of SPENCER[11]. 4 tubes were prepared each containing 150 μC of $Na_2{}^{35}SO_4$. The solution was taken to dryness in a dessicator and 0.02 ml of buffered ATP mixture and 0.08 ml of enzyme were added. After incubation at 37° for 40 min the tubes were heated in a boiling water bath for 1 min, cooled and centrifuged. The combined supernatants and washings were streaked onto acid-washed Whatman 3 MM paper and chromatographed overnight in Solvent A. [^{35}S]PAPS was located with a Geiger

tube, eluted with water (see below under PAPS) and stored at −20°. Some hydrolysis to free [^{35}S]sulphate took place after continual use of such solutions and it was necessary to check the purity at regular intervals by chromatography and automatic scanning. The free sulphate content was calculated from the area under the curve. An accurate estimate of the specific activity could then be made on mixing with purified unlabelled PAPS.

Nucleotides

These were Sigma products with the exception of uridine monophosphates which were obtained from Schwarz Laboratories, New York. The ADP was an unopened sample with a stated purity of 99%.

PAPS

Solutions containing up to 1 μmole of PAPS per ml of incubation mixture were prepared enzymically by the method of BRUNNGRABER[12], using the high-speed supernatant from livers of fasted male rats. Isolation of pure PAPS was achieved by applying 0.5 to 0.8 ml aliquots of the above boiled incubation mixtures as a streak to Whatmann 3 MM paper, previously extracted with dilute HCl and distilled water. Electrophoresis was then carried out in 0.025 M citrate buffer (pH 5.4) for 16 h in the cold room with a voltage gradient of 3 V/cm. Alternatively, the boiled incubation mixture was diluted with an equal volume of water and the nucleotides absorbed by shaking with activated charcoal for 1 h. Elution was effected after 3 separate treatments with 50% (v/v) aqueous ethanol adjusted to pH 9.5 with ammonia, the eluate was concentrated to low volume in vaccuo at 40° and aliquots streaked onto Whatman 3 MM paper*. After electrophoresis, the papers were dried in an air-stream and nucleotides were visualised by examination in ultraviolet light. The zone with the highest mobility, containing the PAPS, was cut out, clamped between glass microscope slides and eluted at 4° with water adjusted to pH 9.5 with ammonia. An equivalent strip of paper from the electrophoretogram was eluted in a similar manner to serve as a blank for analysis. Absorbance was measured at 260 mμ and a molar absorbance index of $15.4 \cdot 10^3$ was used to calculate the concentration of PAPS. The latter was generally about 0.6–1.0 μmole per ml and aliquots gave a single ultraviolet absorbing zone when chromatographed in Solvent A, or subjected to electrophoresis in citrate (pH 5.4). Solutions of purified PAPS were adjusted to pH 8 with ammonia, stored at −20° and used within a week of purification. The concentration of PAPS in the crude preparations was determined by electrophoresis of 0.05 ml aliquots, followed by elution of the PAPS zone.

DEAE cellulose

Whatman DEAE cellulose was suspended in 1 M NaOH and vigorously stirred for 2 h. The material was then washed with distilled water on a large Buchner Funnel until the pH was less than 8. It was then stirred with a saturated solution of Na_2HPO_4 for 30 min and again washed copiously with water until free of excess phosphate.

* Care was needed to ensure that the paper was not overloaded with nucleotide which led to poor separation of PAPS from ATP. Phosphate buffer (pH 6.0) gave better separations but could not generally be used because of the inhibition of the enzyme by phosphate ions.

METHODS

Paper chromatography

The following solvent systems were used in single dimension chromatography employing the descending technique. Proportions are given by vol. A. ethanol–1 M ammonium acetate (pH 7.0; 7.5:3.0). B. di-isopropyl ether–*tert.*-butanol–conc. ammonia–water (6:4:1:9).

Protein

The method of WARBURG AND CHRISTIAN, as adapted by KALCKAR, was used which involves measurement of absorbance at 260 and 280 mμ (ref. 13).

Radioactivity measurements

Liquid scintillation counting employing an Ekco type N664A instrument, was used. 0.5- to 1.0-ml aliquots of aqueous solutions to be counted were mixed with 7 ml of phosphor-detergent mixture composed of toluene phosphor, Triton X-100 and methanol in the ratios 4:3:0.2 by vol. The toluene phosphor contained 8 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2′(5′-phenyloxazolyl)-benzene per l of toluene. Sufficient counts were recorded to allow for an accuracy of 3%.

Scanning of chromatograms

Radioactive components were determined on chromatograms by passing the paper strip through 2 metal plates fitted with adjustable slits. 2 Geiger tubes were mounted vertically over the slits and the pulses were fed to a ratemeter and thence to a recorder. Areas under the curves were determined by wt.

Electrophoretic methods

Acrylamide-gel electrophoresis was performed by the method of ORNSTEIN AND DAVIS[14]. A considerable improvement was affected by eliminating the final sample gel polymerisation step and using instead a mixture of protein, bath-buffer and sufficient Sephadex G-25 to form a firm suspension. In this way dilute proteins could be readily investigated without the need of preliminary concentration.

Isolation of enzyme

Extraction of bovine adrenal glands. The glands were collected at the slaughter-house and transported to the laboratory in ice. After removing adhering fat, the tissue was homogenized in the cold with 2 vol. of 0.01 M phosphate in 0.9% saline (pH 7.4) employing a Waring Blendor. The homogenate was then centrifuged at 100 000 × *g* for 60 min (4°) and the supernatant, containing about 20 mg protein/ml, was collected.

$(NH_4)_2SO_4$ fractionation. In the initial stages of the work satd. $(NH_4)_2SO_4$ sol. was added drop-wise to the high-speed supernatant and protein precipitates were collected at increasing salt concentrations. The enzyme was found to be concentrated in the 0.55 to 0.8 satd. $(NH_4)_2SO_4$ fraction (Fig. 1).

DEAE cellulose chromatography

Convex gradient. A high-speed supernatant was prepared from 300 g of adrenals.

The supernatant (490 ml) was then treated with solid $(NH_4)_2SO_4$ and the fraction precipitating between 0.55 and 0.8 satn. was collected, dissolved in 45 ml of 0.1 M sodium phosphate (pH 7.5) and dialysed overnight against 5 l of the same buffer. After repeating the dialysis against fresh buffer, the protein solution (106 ml containing 17.3 mg/ml) was placed on a column (35 cm × 4 cm) of DEAE-cellulose, previously equilibrated against 0.005 M sodium phosphate (pH 7.5). Two 1 l separating funnels were mounted vertically over the column and the volume in the mixing flask was kept constant (500 ml) whilst 500 ml lots of buffer, of increasing ionic strength, were added to the reservoir. Elution was commenced with 0.005 M phosphate (pH 7.5) in the mixing flask and 500 ml of 0.02 M phosphate (pH 6.5) in the reservoir. Fractions (6 ml) were collected at a flow-rate of 2 ml/min. Aliquots were tested for estrogen sulphotransferase activity as outlined in Fig. 2. Fractions 161–250 were combined, dialysed for 3 days against frequent changes of distilled water (total 20 l) and concentrated by lyophilising to about one third the volume (108 ml, containing 3.4 mg/ml). This solution was stored in ice.

Concave gradient. The 0.55 to 0.8 satd. $(NH_4)_2SO_4$ fraction obtained from 180 g of bovine adrenals was fractionated on a column 40 cm × 44 cm using similar conditions as described above. In this case, however, a concave gradient was used and elution commenced with 500 ml of 0.005 M phosphate (pH 7.5) in the large diameter (15 cm) mixing vessel and 0.02 M phosphate buffer (pH 6.5) in the small diameter (9.5 cm) reservoir. Under these conditions the partially resolved enzyme peaks (Fig. 2a), were completely separated (Fig. 2b). The following fractions were each separately pooled: Fractions 114–115, Fractions 104–124 and Fractions 125–170. These were then dialysed against distilled water (total 25 l) and concentrated by dialysis for 24 h against 10% (w/v) polyethylene glycol 20 000 (Carbowax 20 M), dissolved in 0.005 M Tris–HCl (pH 8.0). Each concentrate was then divided into two parts: one part was stored at −50° and the remainder was dialysed against distilled water and lyophilised.

Standard enzyme assay. Optimum conditions for assay of the enzyme, in which both substrates were at saturation level, was achieved in the following incubation mixture: 0.25 mM PAPS; 10^5 counts/min [^{35}S]PAPS; 0.08 mM estrone added as 5 μl of a propylene glycol stock solution; 20 mM $MgCl_2$; 0.1 M Tris–HCl buffer (pH 8.1); enzyme 50–200 μg. Total vol. 0.135 ml. Any departures from these conditions were noted under the appropriate experiments. Incubation was carried out for 20 min at 37° and the reaction stopped by placing the tubes in a boiling water bath for 1 min. Estimation of the steroid sulphate in the solution was then carried out by the procedure of WENGLE[15]. 1 ml of 0.1 M $Ba(OH)_2$ was added to each tube followed by 1 ml of 0.05 M H_2SO_4. After mixing, the heavy precipitate containing inorganic sulphate and PAPS, was centrifuged off and the supernatant decanted into 10 ml centrifuge tubes. CO_2 gas was bubbled into the solution to remove excess Ba^{2+}, the precipitate centrifuged down and 1 ml of the supernatant used for measurement of [^{35}S]steroid sulphate by liquid scintillation counting. A blank, employing 5 μl of propylene glycol in place of estrogen, was also incubated and the recorded counts were subtracted from each individual determination. The total counts, representing ester sulphate, were then converted to mμmoles from a knowledge of the specific activity of the [^{35}S]PAPS.

Assay employing paper chromatography. The WENGLE method could not be used

for assay of estrogen sulphotransferase in the crude adrenal extracts due to the formation of an unknown radioactive product in addition to estrone [^{35}S]sulphate. This unknown product was not removed by the treatment with $Ba(OH)_2$ and H_2SO_4. Use of the WENGLE method was justified with enzyme purified on DEAE-cellulose columns since this unknown radioactive product was not formed with these preparations, as witnessed by paper chromatography and autoradiograms. Solvent system B effectively separated estrogen sulphates from the unknown product and scanning such chromatograms enabled the amounts of the two products to be determined. When chromatographic methods were used, the specific activity of the [^{35}S]PAPS was increased 5-fold.

Enzyme unit. This was defined as that quantity of enzyme which synthesised 1 mμmole of estrone sulphate in 20 min at 37° under conditions of incubation given above.

RESULTS

Fractionation of the enzyme

As evident from Fig. 1, estrogen sulphotransferase activity first appeared in the 0.55 satd. $(NH_4)_2SO_4$ fraction. A separate phenol sulphotransferase was also present in the adrenal extract which appeared in optimal concentration in the 0.45 and 0.55 satd. $(NH_4)_2SO_4$ fractions when *p*-nitrophenol was used as test substrate. This enzyme was not present in the 0.6 and 0.8 satd. fractions. DEAE-cellulose chromatography resolved estrogen sulphotransferase into two separate enzyme activities which were partially separated using a convex phosphate gradient and completely resolved when a concave gradient was employed (Fig. 2). Both enzyme fractions yielded estrone [^{35}S]sulphate on incubation with estrone and [^{35}S]PAPS. The first fraction appearing from the column will be referred to as Fraction A and the second peak as Fraction B. The former was the more stable, losing only a small percentage of its activity on standing in ice for a period of 2 weeks. The B form however lost 50% of its initial activity in this time.

Overall purification

When the estrogen sulphotransferase activity in the crude supernatant, determined by paper-chromatographic methods, was compared with the activity of the 0.55–0.8 satd. fraction, determined by the standard assay procedure, the latter fraction was found to contain 4 to 6 times as many enzyme units as the original extract. Examination of the products at the end of the incubation period showed that no [^{35}S]PAPS remained using crude extracts, but excess [^{35}S]PAPS was still present in the case of the $(NH_4)_2SO_4$ fraction. SUZUKI AND STROMINGER[16] described the rapid hydrolysis of PAPS to free sulphate by a sulphatase present in hen oviducts. The sulphatase was inhibited by 0.01 M fluoride and 0.01 M phosphate. When these additions were made to the estrogen sulphotransferase assays, the number of enzyme units in the $(NH_4)_2SO_4$ fraction were still far in excess of those present in the crude extract. Addition of K_2SO_4 up to a concentration of 0.05 M did not inhibit the sulphatase sufficiently, and increasing the concentration of PAPS still did not enable accurate enzyme determinations to be made on the crude extract. An approximate assessment of the overall purification achieved could be made by assuming that the

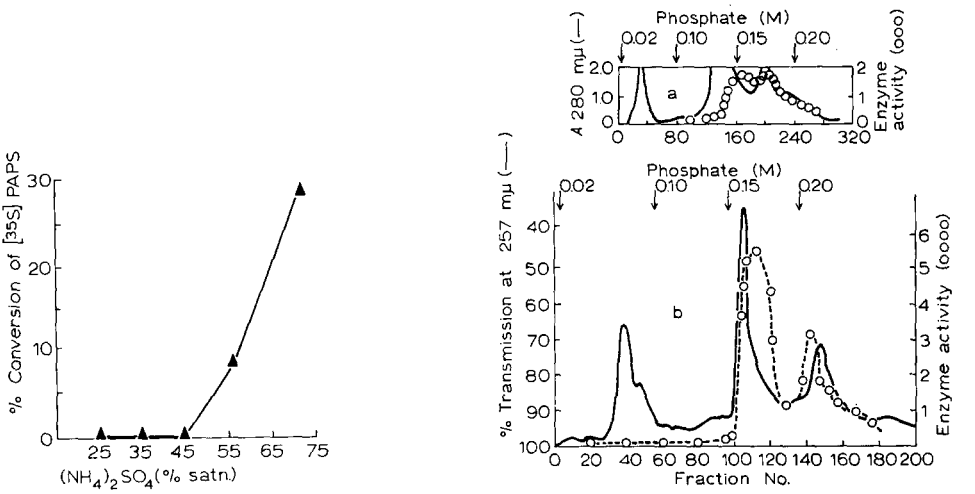


Fig. 1. Formation of estrone sulphate by successive $(NH_4)_2SO_4$ fractions. Each fraction (see METHODS), derived from 12 ml of supernatant, was dissolved in 2 ml of 0.1 M phosphate (pH 7.5). Incubation mixtures contained 0.025 ml of crude PAPS (17 mμmoles), 0.02 ml of 0.1 M phosphate (pH 7.5), 0.005 ml of [^{35}S]PAPS ($3 \cdot 10^4$ counts/min), 0.005 ml of a propylene glycol solution of estrone (final concn. 0.2 mM) and 0.05 ml of enzyme. Incubation was carried out for 1 h at 37° and the labelled products separated by paper chromatography in Solvent B and quantitated by scanning. Labelled products, derived by sulphation of endogenous acceptors, were formed with each enzyme fraction.

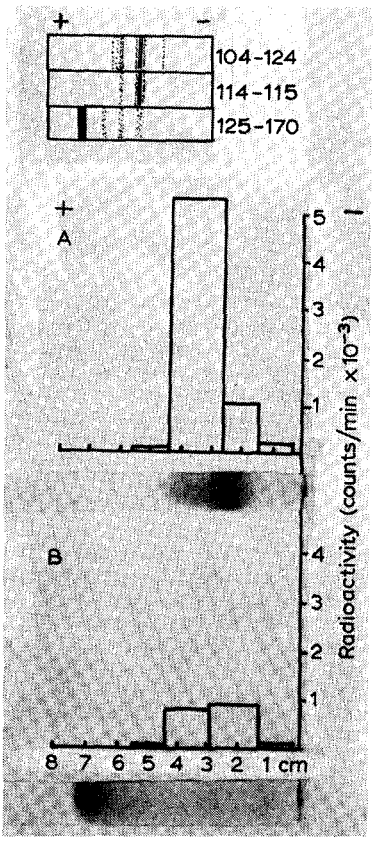
Fig. 2. Resolution of estrogen sulphotransferase on DEAE-cellulose. The 0.55 to 0.8 satd. $(NH_4)_2SO_4$ fraction was chromatographed using convex gradient elution in (a), and concave elution in (b). Protein concn. was determined in (a) by direct measurement of absorption at 280 mμ on the eluted fractions. In (b), the eluate was passed through an LKB optical unit (Type 4701A) fitted with a mercury lamp and coupled to an LKB recorder (Type 6520A). Arrows indicate the fraction number at which the phosphate buffers of increasing molarity were added to the top vessel in (a), or the small diameter reservoir in (b). Enzyme assay was carried out as follows. Each incubation contained 0.1 ml of eluted fraction, 0.01 ml of crude PAPS (5 mμmoles), 0.01 ml [^{35}S]PAPS ($1 \cdot 10^5$ counts/min), 0.005 ml estrone solution (0.1 mM final concn.) and 0.05 ml of 0.2 M Tris–HCl (pH 8.1). After 1 h at 37°, the enzyme activity was measured as stated under standard enzyme assay (METHODS).

bulk of the enzyme was precipitated in the 0.55–0.8 fraction (see Fig. 1). On average, the weight of the protein precipitating in this fraction was 15% of the total protein present in the crude supernatant. The purification at this stage was then 7-fold. The average specific activity of the enzyme obtained from the DEAE cellulose was 34 as compared to 6 for the $(NH_4)_2SO_4$ fraction, giving an overall purification of about 35-fold. The recovery of enzyme in the chromatography step was 60%.

Identification of product

Fraction A was used in all experiments described below under their appropriate headings. When incubated with [^{14}C]estrone and cold PAPS, or [^{35}S]PAPS and cold estrone, the enzyme gave products which behaved identically on paper chromatography. Isolation of the products was achieved initially by chromatography in Solvent B. Inorganic sulphate and PAPS remain at the origin in this solvent system and estrone sulphate has an R_F of about 0.3 (see ref. 17). After elution, the two isotopically-labelled products behaved identically to authentic estrone sulphate,

when chromatographed in other solvent systems[17]. Autoradiographs revealed that a single sulphated product was formed when 17 β-estradiol and estriol were used as substrates and paper chromatography was carried out in Solvent B. After elution, the radioactive species were shown to have a nett charge of -1 on paper electrophoresis. Thus a monosulphated product was formed in each case.



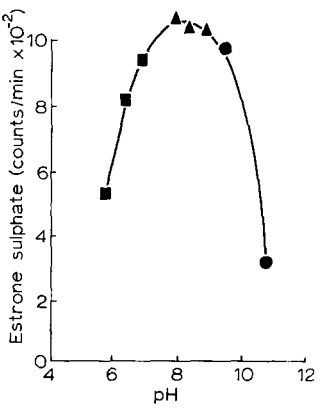


Fig. 3. Electrophoretic behaviour of the A and B fractions on acrylamide gel (top) and paper (bottom). Fractions A and B represent pooled fractions 104–124 and 125–170, of Fig. 2, respectively. Paper electrophoresis was carried out in 0.025 M barbiturate HCl buffer (pH 8.6) for 5 h at 12 V/cm in the cold room. Lyophilised preparations (1 mg) of A and B were employed. The papers were cut lengthwise into 2 equal strips. One strip was stained with 1% (w/v) Amido Black and the companion strip was divided into 1 cm segments and these extracted for 1 h with 0.3 ml of 0.1 M Tris–HCl (pH 8.1). An aliquot (0.1 ml) was added to 0.1 ml of buffered estrone–[^{35}S]PAPS mixture of a similar composition to that given in Fig. 2 and the enzyme assayed in a like manner.

Fig. 4. pH optimum of the enzyme. Standard enzyme assay using Fraction A. ■, Tris–maleic acid–NaOH. ▲, Tris–HCl. ●, glycine–NaOH. Final buffer concns. were 0.19 M in each case.

Electrophoretic properties

The behaviour of enzyme fractions A and B on electrophoresis on paper and acrylamide gel is shown in Fig. 3. Enzyme activity was demonstrated in the same

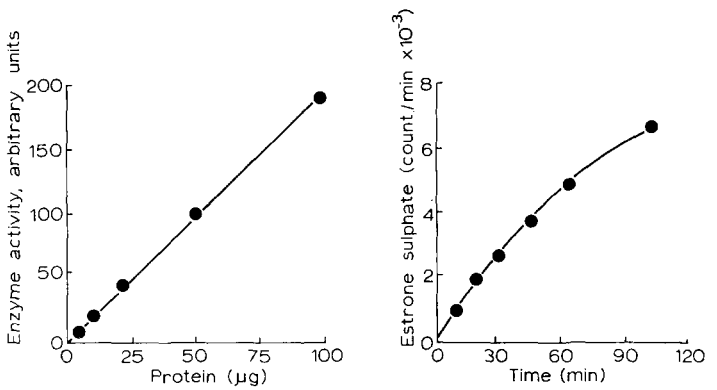


Fig. 5. Effect of enzyme concn. and time of incubation. Standard enzyme assay was employed using a lyophilised preparation of Fraction A.

region of the paper in both fractions. The major protein band present in Fraction B did not possess enzyme activity.

pH optimum

A pH optimum in the vicinity of 8.0 was obtained as shown in Fig. 4.

Effect of protein concentration and time of incubation

The increase in activity with increasing protein is shown in Fig. 5. Zero order kinetics was obeyed for incubation periods of some 30 min (Fig. 5).

SH-blocking agents

The enzyme was unaffected by low concentrations of SH-blocking agents such as *p*-chloromercuribenzoate but was sensitive at concentrations near 1 mM (Table I).

Effect of cysteine

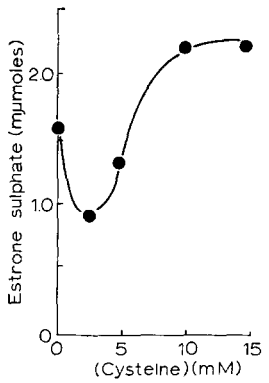
Fraction A enzyme was activated by addition of cysteine; the actual degree of activation depending on the particular enzyme preparation. A preparation of Fraction A, which had been dialysed against water and then lyophilised, was activated to the

TABLE I

EFFECT OF SH-BLOCKING AGENTS ON ENZYME ACTIVITY

PAPS concn. 0.1 mM, otherwise standard assay procedure employed. Enzyme: lyophilised Fraction A, 200 μg. Addition of the SH-blocking agent was made prior to addition of enzyme.

Substance	*Concn. (mM)*	*Inhibition (%)*
p-Chloromercuribenzoate	0.02	0
	1	43
	10	80
o-Iodosobenzoate	1	7
	10	60



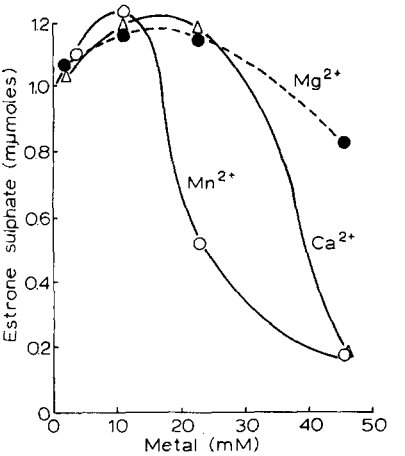


Fig. 6. Effect of cysteine addition on enzyme activity. Standard enzyme assay with the exception that the PAPS concn. was 0.13 mM. Enzyme: lyophilised Fraction A (200 μg protein).

Fig. 7. Effect of metal ions on enzyme activity. Standard enzyme assay. Enzyme: combined A and B fractions isolated as in Fig. 2a and stored frozen, 0.025 ml (84 μg protein).

TABLE II

EFFECT OF METAL IONS ON ENZYME ACTIVITY

Expt. 1. PAPS 0.10 mM; estrone 0.1 mM; enzyme: lyophilised Fraction A, 300 μg. The concentration of metal ion was 11 mM. Expt. 2. PAPS 0.13 mM; estrone 0.1 mM; cysteine 13 mM; enzyme: Fraction A dialysed overnight against 1 mM EDTA, 200 μg. The concentration of metal ion was 21 mM. When used in combination, the concentration of each separate ion was 21mM. The effect of EDTA shown below was tested at a final concn. of 10 mM.

Expt.	*Addition divalent ion*	*Activation or inhibition (%)*
1	Mg	+ 53
	Mn	+ 44
	Co	− 70
	Zn	−100
2	Mg	+ 47
	Ca	+ 35
	Mn	+ 18
	Co	− 26
	Zn	− 80
	Ni	− 91
	Mg + Ca	+ 37
	Mg + Mn	− 22
	Mg + Co	− 47
	Mg + Zn	− 93
	Mg + Ni	− 91
	EDTA	No effect

extent of 190% of the control by addition of 10 mM cysteine. The effect of increasing cysteine concentration on activity using the same enzyme fraction, but lyophilised on a separate occasion, is shown in Fig. 6.

Metal ions

Since the phosphokinase enzymes, as a group, either possess an absolute requirement for, or show an enhanced activity in the presence of certain divalent metal ions, the effect of metal ions on the sulphotransferase was examined. Table II shows that Mg^{2+}, Mn^{2+} and Ca^{2+} were able to activate the reaction, whilst Zn^{2+}, Co^{2+} and Ni^{2+} were inhibitory. Some degree of precipitation occurred in all the incubations with the exception of Mg^{2+}. Inhibition was found to occur when high concentrations of Mg^{2+}, Ca^{2+} and Mn^{2+} were employed; this inhibition being more pronounced with the latter two metal ions (Fig. 7).

The effect of increasing metal ion concentration on enzyme activity in the presence of 15 mM cysteine is shown in Fig. 8. Activation by Mn^{2+}, but not Mg^{2+}, is

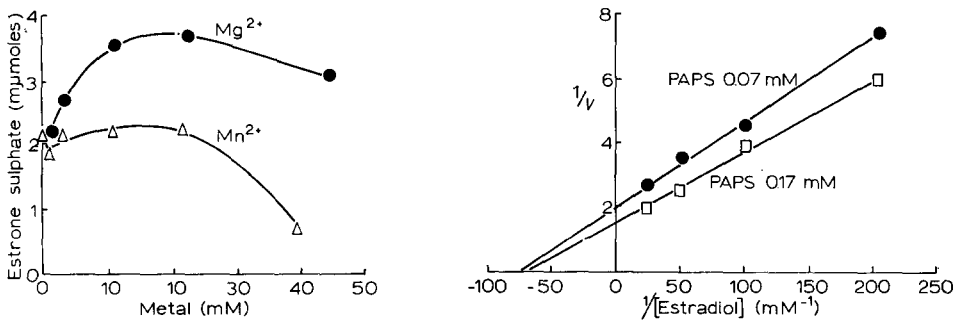


Fig. 8. Effect of metal ions in the presence of 15 mM cysteine. Enzyme: Fraction A, stored frozen, was thawed and dialysed overnight against 1 mM EDTA made up in 5 mM Tris–HCl (pH 7.7). Volume of enzyme used was 0.03 ml (156 μg protein).

Fig. 9. Effect of varying 17 β-estradiol at fixed levels of PAPS using the A form of the enzyme. Standard enzyme assay using the PAPS concentrations shown and 0.005 ml aliquots of stock dilutions of the estrogen.

virtually eliminated due evidently to chelation of the cysteine by the Mn^{2+}, since pronounced colours were observed with the latter ions. Other effects of metal ions, and combinations of metal ions, are shown in Table II.

Inhibition by nucleotides

Table III shows the effect of various nucleotides on the enzyme activity. ADP alone was inhibitory at 1 mM concentration and possessed the highest inhibitory action when the experiment was repeated at nucleotide concentrations of 10 mM. The inhibition by ADP was non-competitive (Fig. 10).

Specificity

Fraction A enzyme was tested with a number of different substrates under standard enzyme assay conditions, with the exception that the incubation time was

TABLE III

EFFECT OF NUCLEOTIDES ON ENZYME ACTIVITY

PAPS 0.18 mM; 17 β-estradiol 0.1 mM; enzyme: lyophilised Fraction A, 300 μg.

Nucleotide	*Inhibition (%)*	
	*1 mM**	*10 mM*
3'-AMP		30
5'-AMP		26
5'-ADP	24	86
5'-ATP	—	70
3',5'-AMP		—
Adenosine		—
3'-UMP		—
5'-UMP		14
5'-UTP	—	64
5'-GMP		—
5'-IMP		18
Na_3PO_4	—	14

* No inhibition was found with any other nucleotide tested at this concentration. A dash indicates not tested.

extended to 1 h. The following compounds, at concentrations of 1.5 mM, failed to act as substrates under the above conditions: dehydroepiandrosterone, phenol, *p*-nitrophenol, α-naphthol, β-naphthol, β-naphthylamine and 17 β-estradiol-3-methyl ether. Stilbestrol and hexestrol, but not dienestrol, gave low but reproducible rates of sulphation. Under the above conditions (PAPS 0.23 mM) these synthetic estrogens were sulphated to the extent of 7% as compared to the sulphation of estrone. The sulphation of stilbestrol and hexestrol, and the apparent non-sulphation of phenol and dienestrol, was checked in another experiment which was made more sensitive

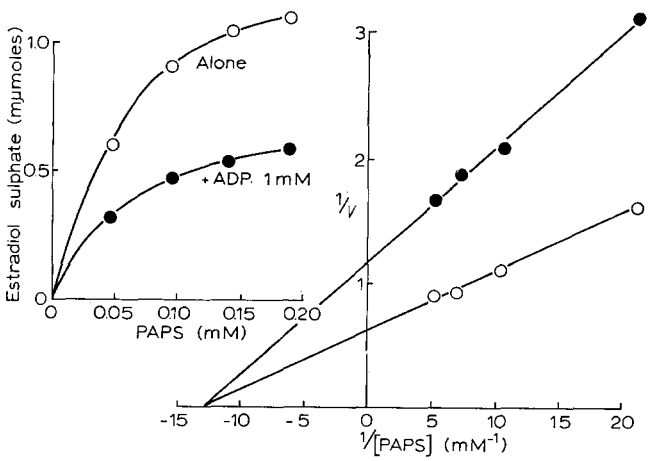


Fig. 10. Non-competetive inhibition of PAPS by ADP. PAPS was varied in the presence of a constant level of 17 β-estradiol (0.1 mM). Mg^{2+} was omitted from the incubations. Enzyme: lyophilised Fraction A (300 μg protein).

TABLE IV

SULPHATION OF SYNTHETIC ESTROGENS

PAPS 0.007 mM; [^{35}S]PAPS increased to $4 \cdot 10^5$ counts/min. Propylene glycol solutions of substrates (final concn. 0.15 mM) were employed except in the case of phenol, when aqueous solutions were used. Enzyme: lyophilised Fraction A, 300 μg. Incubation time 1 h.

Substrate	*"Ester-sulphate"* total counts/min*
Propylene glycol (control)	2 200
Stilbestrol	4 830
Dienestrol	2 670
Hexestrol	5 460
Phenol	1 790
Estrone	201 600

* The soluble fraction remaining after removal of [^{35}S]sulphate and [^{35}S]PAPS by addition of H_2SO_4 and $Ba(OH)_2$ (see METHODS).

by increasing the specific activity of the [^{35}S]PAPS. As can be seen in Table IV, the above results are confirmed, although the rate of sulphation of stilbestrol and hexestrol compared to estrone is somewhat lower in this case, possibly due to the greatly lowered PAPS concentration employed.

Reaction rates of natural estrogens

Comparison was made of the rate of sulphation of the three classical estrogens under standard assay conditions, at substrate concentrations of 0.1 mM. Rates, compared to estriol as 1.0, were: estriol 1.0, 17 β-estradiol 0.6, estrone 0.53 Introduction of a keto group at position 16 in 17 β-estradiol (estratriene-3-hydroxy-17 β-hydroxy-16-one) did not significantly alter the rate of sulphation. Equilenin was sulphated at a rate of 0.42 times that of estriol, indicating that the presence of a saturated ring B was not essential for enzyme activity.

Kinetic studies

The specificity of the estrogen sulphokinase indicated that it was free of contaminating phenol and steroid alcohol sulphotransferase. Using the A enzyme, normal Michaelis–Menten kinetics were observed when estrogen was varied in the presence of fixed but differing concentrations of PAPS. The apparent K_m for 17 β-estradiol (14 μM) was unaffected by the PAPS concentration (Fig. 9), indicating that a sequential rather than a ping-pong mechanism was operating[18]. The apparent K_m for PAPS in the presence of saturating levels of 17 β-estradiol was found to be 70 μM (Fig. 10). ADP proved to be a non-competitive inhibitor (Fig 10) with a K_i value of 1.1 mM. These values did not alter significantly in the presence of Mg^{2+}.

Kinetic studies with the B form of the enzyme proved to be complex and the nature of the relationship between the A and B forms is dealt with in Part IV (ref. 19).

DISCUSSION

The preparation of the enzyme, free of other contaminating sulphotransferase enzymes, represents the first steroid sulphotransferase to be isolated in comparatively

pure form. It is of interest then to compare the sulphotransferase with the phosphokinases, as a group, because of certain similarities in their behaviour *e.g.*, activation by metal ions, sensitivity to SH-blocking agents, *etc.* The pH optimum of 8.0 is close to that of many phosphokinases which usually show pH optima in the range 6.5–9.0 (ref. 20). A close analogy also occurs as regards metal ion activation. In general Mg^{2+} and Mn^{2+} activate phosphotransferases but not phosphorylases[21]. Ca^{2+} will sometimes activate, *e.g.* creatine phosphokinase, but in other cases Ca^{2+} acts as an antagonist for Mg^{2+} (see ref. 21). The analogy of estrogen sulphotransferase to creatine phosphokinase is also demonstrated by the fact that both enzymes are inhibited by Zn^{2+} (ref. 21 and Table II).

The activation by metals shown in Fig. 7 bears a striking resemblance to the figure illustrated in DIXON AND WEBB[22] for the metal-ion activation of phosphokinases which is stated to be typical of activation curves for such enzymes. Essential features of both figures are the formal resemblance to Michaelis curves with the velocity then decreasing at higher concentrations of metal ion. Mn^{2+} reaches its optimum concentration at lower values than Mg^{2+}, and the reduction in velocity occurs at lower Mn^{2+} concentrations in both instances. Most phosphokinases show an absolute requirement for metal ions and very low activity is obtained in the absence of added metal ion. This residual activity has been explained by ENNOR AND MORRISON[23] to be due to the presence of traces of metal ion remaining in the enzyme preparation. Residual activity can be removed by addition of complexing agents to the incubation medium. The enzyme may show a high affinity for the metal once it is added to the medium, *e.g.* in the case of arginine phosphokinase an EDTA:Mg^{2+} ratio of 2:1 was required to completely abolish enzyme activity, although the enzyme showed very low activity in the absence of added metal[24]. It is the opinion of NORDLIE AND LARDY[20] that absence of stimulation by added metal cannot be interpreted as an absolute nonrequirement for metal ion, since the isolated enzyme may contain sufficient tightly bound metal to satisfy its requirements in this respect. There has been no direct evidence for this amongst phosphokinases, although a recent paper on the enzyme acid phosphatase, has shown it to contain Mg^{2+} as an essential metal ion which is bound to an aspartic acid moiety[25]. ORSI AND SPENCER established that the enzyme choline sulphotransferase, which has a pH optimum of 8.0, does not possess even a partial requirement for Mg^{2+}. EDTA (10 mM) had no effect on reaction rate[26]. A similar type of behaviour was described for the phenol sulphotransferase isolated from guinea-pig liver by BANERJEE AND ROY[9], although the partially-purified steroid sulphotransferase, isolated from the same source, was activated by some 40% on addition of Mg^{2+}. The presence of tightly bound metal could be the explanation for the behaviour exhibited by the sulphotransferase although the complete ineffectiveness of EDTA to suppress activity when added to the enzyme, or when the enzyme was dialysed against dilute EDTA solutions (Table II), casts considerable doubt on this possibility. Since Mg^{2+} was observed to have no effect on the K_m value for PAPS with estrogen sulphotransferase, the metal may then increase reaction rate by screening the negative charges on the PAPS when the latter is bound to the enzyme, thus facilitating nucleophilic attack on the S atom by the phenolic hydroxyl group of the estrogen.

Inhibition of estrogen sulphotransferase by SH-blocking agents, as shown in Table I, implicates the importance of a free thiol group or groups, in the maintenance

of enzyme activity. Choline sulphotransferase is inhibited almost completely by 0.8 mM concentrations of *p*-chloromercuribenzoate[26], and a requirement for SH compounds to achieve full activity in the case of phenol sulphotransferase, has been reported[27]. This property of sulphotransferase parallels the behaviour of the phosphokinases but the danger of assuming that a SH group forms part of the active site of an enzyme, based on inhibition studies with phenyl-mercurials *etc.*, has been pointed out by BOYER[28]. Indeed, in the case of rabbit muscle myokinase, both SH groups react stoichiometrically with *p*-chlormercuribenzoate to form a complex which retains 45% of the initial activity[29]. This suggested that the SH groups were near, rather than part of, the active site and were perhaps responsible for maintaining the proper conformation of the protein[29].

It seems firmly established that the enzyme is specific for natural estrogens. The 3-hydroxyl appears to be the only group attacked since a single sulphated product was formed with such estrogens as 17 β-estradiol, and furthermore, the 3-methyl ether of the latter gave no product whatever. Although the existence of a phenol sulphokinase which would not sulphate estrone or dehydroepiandrosterone had been established[9,30], the existence of an enzyme, specific for estrogens, had not previously been demonstrated. Kinetic studies on Form A of estrogen sulphotransferase showed that the binding of one substrate was independent of the concentration of the other which was in keeping with recent studies on choline sulphotransferase[26] and phenol sulphotransferase[9]. For the latter enzyme, the mechanism has been established as a rapid equilibrium random bi–bi reaction[31].

A point of considerable interest was the weak but reproducible sulphation of stilbestrol and hexestrol. Although dienestrol possesses a biological activity of the same order as the latter synthetic estrogens[32], it was not sulphated by the enzyme at a detectable rate. Stilbestrol exists in the trans configuration, as evidenced by X-ray crystallographic studies, and it has been shown by such means that stilbestrol and estrone have the same overall dimensions[32]. Both stilbestrol and hexestrol must possess a conformation which is similar to the natural estrogens, and results in the ability of these substances to bind to the estrogen sulphotransferase. Despite the inability of the enzyme to sulphate the physiologically active synthetic estrogen dienestrol, the enzyme may be potentially useful as a tool for the rapid screening of compounds for potential estrogenic action—either by way of their ability to act as sulphate acceptors, or perhaps as competetive inhibitors. Either type of activity could perhaps indicate that the given compound was capable of assuming a conformation closely resembling a natural estrogen and therefore potentially capable of binding at the specific target site.

REFERENCES

1 H. I. CALVIN, R. L. VANDEWIELE AND S. LIEBERMAN, *Federation Proc.*, 22 (1963) 531.
2 K. D. ROBERTS, L. BANDI, H. I. CALVIN, W. D. DRUCKER AND S. LIEBERMAN, *J. Am. Chem. Soc.*, 86 (1964) 958.
3 P. K. SIITERI AND P. C. MACDONALD, *Steroids*, 2 (1963) 713.
4 E. E. BAULIEU AND F. DRAY, *J. Clin. Endocrinol. Metab.*, 23 (1963) 1300.
5 A. AAKVAAG, A. A. HAGEN AND K. B. EIK-NES, *Biochim. Biophys. Acta*, 86 (1964) 622.
6 E. E. BAULIEU, *J. Clin. Endocrinol. Metab.*, 22 (1962) 501.
7 R. G. WIELAND, R. P. LEVY, D. KATZ AND H. HIRSCHMANN, *Biochim. Biophys. Acta*, 78 (1963) 566.
8 Y. NOSE AND F. LIPMANN, *J. Biol. Chem.*, 233 (1958) 1348.

9 R. K. BANERJEE AND A. B. ROY, *Mol. Pharmacol.*, 2 (1966) 56.
10 J. B. ADAMS, *Biochim. Biophys. Acta*, 82 (1964) 572.
11 B. SPENCER, *Biochem. J.*, 77 (1960) 294.
12 E. G. BRUNNGRABER, *J. Biol. Chem.*, 233 (1958) 472.
13 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic, New York, 1957, p. 447.
14 L. ORNSTEIN AND B. J. DAVIS, *Disc Electrophoresis*, Distillation Products Industries, Rochester, N.Y., 1962.
15 B. WENGLE, *Acta Chem. Scand.*, 18 (1964) 65.
16 S. SUZUKI AND J. L. STROMINGER, *J. Biol. Chem.*, 235 (1960) 267.
17 J. J. SCHNEIDER AND M. L. LEWBART, *Recent Progr. Hormone Res.*, 15 (1959) 201.
18 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 104.
19 J. B. ADAMS AND M. CHULAVATNATOL, *Biochim. Biophys. Acta*, 146 (1967) 509.
20 R. NORDLIE AND H. LARDY, in P. D. BOYER, H. LARDY AND K. MYRBACK, *The Enzymes*, Vol. 6, Academic, New York, 1962, p. 4.
21 M. DIXON AND E. C. WEBB, *Enzymes*, Longmans, London, 2nd Ed., 1964, p. 328.
22 M. DIXON AND E. C. WEBB, *Enzymes*, Longmans, London, 2nd Ed., 1964, p. 424.
23 A. H. ENNOR AND J. F. MORRISON, *Physiol. Rev.*, 38 (1958) 631.
24 J. F. MORRISON, D. E. GRIFFITHS AND A. H. ENNOR, *Biochem. J.*, 65 (1957) 143.
25 H. ALBERS, G. BÜSING AND G. SHUDT, *Enzymologia*, 30 (1966) 149.
26 B. A. ORSI AND B. SPENCER, *J. Biochem. (Tokyo)*, 56 (1964) 81.
27 J. D. GREGORY AND F. LIPMANN, *J. Biol. Chem.*, 229 (1957) 1081.
28 P. D. BOYER, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 1, Academic, New York, 1959, p. 511.
29 L. F. KRESS, V. H. BONO AND L. NODA, *J. Biol. Chem.*, 241 (1963) 2293.
30 J. B. ADAMS, *Biochim. Biophys. Acta*, 101 (1963) 478.
31 R. K. BANERJEE AND A. B. ROY, *Biochem. J.*, 100 (1966) 59P.
32 J. JACQUES, *Soc. Chim. France Bull.*, 16 (1949) 411.