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ENZYMIC SYNTHESIS OF STEROID SULPHATES

V. ON THE BINDING OF ESTROGENS TO ESTROGEN SULPHOTRANSFERASE

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SUMMARY

1. Estrone has been shown to be bound to the estrogen sulphotransferase (3′-phosphoadenylylsulphate:estrone sulphotransferase, EC 2.8.2.4) of bovine adrenals following purification steps involving $(NH_4)_2SO_4$ precipitations and DEAE-cellulose chromatography. Incubation of the purified enzyme with [^{35}S]adenosine-3′-phosphate-5′-phosphosulphate led to the liberation of estrone [^{35}S]sulphate which was characterised by chromatography and co-crystallisation with authentic material. Other estrogens were not detected.

2. The binding of estrogens to the A form of the enzyme has been examined by a determination of K_m and $v_{max.}$ values. 17-Deoxyestrone acted as a substrate with a K_m of 14 μM which was identical to the value for other estrogens containing hydroxyl groups on the β-side of ring D. Variation in $v_{max.}$ values amongst this group did, however, occur.

3. Introduction of α-hydroxyl groups into ring D decreased the K_m values.

4. An overall fit of the steroid *via* the rear or α-side to the protein surface is judged to be the best interpretation of these results which is also in keeping with other studies on steroid binding to proteins. Multi-point attachment by way of Van Der Waals forces could be assisted by polar attachments *via* α-hydroxyl groups on ring D.

INTRODUCTION

In the previous paper (Part IV)[1], estrogen sulphotransferase (3′-phosphoadenylylsulphate:estrone sulphotransferase, EC 2.8.2.4), Form B, was shown to exhibit a difference in behaviour as regards the sulphation of the three classical estrogens. Each of the three estrogens yielded a sigmoid-shaped velocity–substrate

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

curve at low substrate levels, but at higher concentrations the sulphation of estrone was observed to undergo an increased rate, whereas the sulphation of 17 β-estradiol and estriol was inhibited. It has now been found that estrone is present in the purified enzyme and is released as estrone sulphate on incubation with adenosine-3'-phosphate-5'-phosphosulphate (PAPS). The finding of bound estrone on the enzyme has prompted a study of the requirements for the binding of other estrogens to the enzyme. In keeping with previous studies on the binding of steroids to proteins[2], results were perhaps best interpreted as implicating the α-side of the estrogen as the site of binding to the enzyme.

MATERIALS

In general the materials and methods were those described in Parts III and IV[1,3]. 16-Epiestriol and 17-deoxyestrone were kindly supplied by Professor G. F. MARRIAN and Dr. A. B. ROY, respectively. 17-Epiestriol was obtained from the Medical Research Council, Steroid Reference Collection, London. Potassium estrone sulphate was purchased from Sigma Chemical Co.

METHODS AND RESULTS

Paper chromatography systems

Two-dimensional System A: first direction, phenol–water (400:100, w/w) in an atmosphere of NH_3; second direction, butanol satd. with 2 M NH_4OH. System B: isopropyl ether–*tert.*-butanol–conc. ammonia–water (6:4:1:9, by vol.). System C: 0.4 M potassium phosphate (pH 6.5).

Demonstration of enzymically bound estrone

When the associated form of estrogen sulphotransferase, obtained by isolation in the presence of mercaptoethanol, was incubated with [^{35}S]PAPS of high specific activity a number of radioactive products were formed. These were separated by paper chromatography in Solvent B (Fig. 1). One of these radioactive components (P) possessed an R_F identical to that of estrone or estradiol sulphates, and the major product (Q) had an R_F identical to that of estriol sulphate, in this solvent system. On examination in a two-dimensional system, the first-mentioned Component P behaved as estrone or estradiol sulphate but the major Component Q did not migrate into the 'steroid sulphate' region of the chromatogram[4]. Further indications that the Component P may have been an estrogen sulphate was obtained by adding estrone to the incubation mixture. Following two-dimensional chromatography, an increase in radioactivity in this zone on the autoradiograph was obtained, *i.e.* the presence of an additional zone was not observed (Fig. 1). Zones P and Q were eluted and rechromatographed in a buffer system designed for separating the sulphate esters of the classical estrogens[5]. It can be seen from Fig. 1 (Solvent C) that Compound P had an R_F identical to estrone sulphate. Compound Q did not migrate into the estrogen sulphate area.

When the non-associated form of the enzyme was incubated with [^{35}S]PAPS of high specific activity then a single radioactive product was obtained which had an R_F identical to estrone sulphate (Fig. 2). This preparation (Fraction A_2) of enzyme

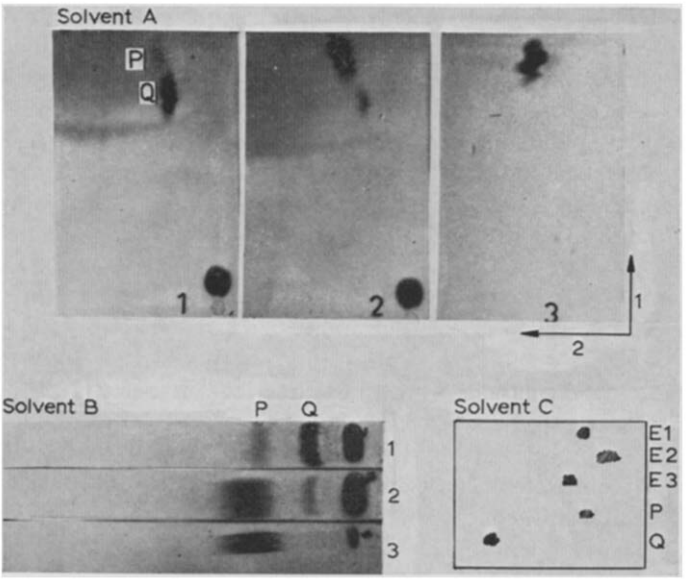


Fig. 1. Chromatographic identification of estrone [^{35}S]sulphate produced on incubating enzyme with [^{35}S]PAPS. The incubation contained 0.05 M Tris–HCl (pH 8.1), 20 mM Mg^{2+}, [^{35}S]PAPS, $2.5 \cdot 10^4$ counts/min and enzyme (prepared and purified on DEAE-cellulose in the presence of mercaptoethanol[1]), 0.05 ml (330 μg protein). Total vol. was 0.17 ml. Incubation was for 1 h at 37° when the reaction was stopped by placing in a boiling water bath for 1 min. Aliquots were chromatographed and autoradiographs prepared. 1, [^{35}S]PAPS and enzyme; 2, estrone (0.05 mM) added; 3, authentic [^{14}C]estrone sulphate; E1, E2 and E3, estrone-3-sulphate, 17 β-estradiol-3-sulphate and estriol-3-sulphate, respectively.

was obtained after DEAE-cellulose chromatography and $(NH_4)_2SO_4$ fractionation. It was a relatively pure sample of estrogen sulphotransferase and its behaviour on gel electrophoresis has been shown previously (Fig. 7, Part IV[1]). The associated form of the enzyme which gave rise to Compounds P and Q above (Fig. 1) contained a major protein contaminant (see Fig. 7, Part IV[1]). This major contaminant did not possess estrogen sulphotransferase activity[1]. By separating the major protein contaminant from the estrogen sulphotransferase by paper electrophoresis* in 0.05 M

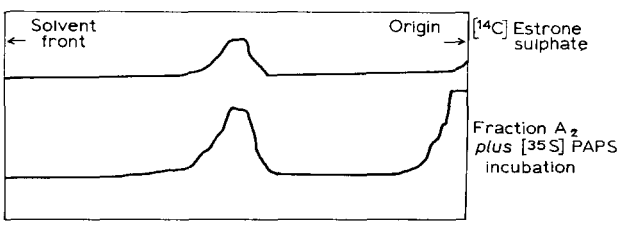


Fig. 2. Single product obtained on incubation of Fraction A_2 enzyme[1] with [^{35}S]PAPS under similar conditions to those in Fig. 1. Paper chromatography in Solvent B; radioactivity recorded with a Nuclear Chicago Actigraph II chromatogram scanner. Radioactivity at the origin represents [^{35}S]PAPS and [^{35}S]sulphate.

* Conditions used for this experiment were essentially those described previously[3] except that estrogen was not added to the incubations and the latter were carried out for 16 h at 37° and radioactive ester sulphate estimated by liquid scintillation counting[3].

barbiturate–HCl buffer (pH 8.6) it was found that this protein yielded the Product Q on prolonged incubation with [^{35}S]PAPS. This explained the lack of formation of the Product Q on incubation of Fraction A_2, since the latter did not contain the protein referred to above.

Final proof of the identity of the Product P, as estrone sulphate, was obtained by co-crystallisation with authentic material. These results are shown in Table I.

TABLE I

IDENTIFICATION OF ENZYMICALLY BOUND ESTRONE

The peak corresponding to estrone sulphate in Fig. 2 was eluted from the paper with water (50 ml). Potassium estrone sulphate (29 mg) was added and the quinidine salt was precipitated by addition of a warm satd. aqueous solution of quinidine sulphate (British Drug Houses). The precipitate was collected by filtration and thoroughly washed with water. After drying in a dessicator, the weighed sample was dissolved in methanol (3 ml) and an aliquot removed for counting. The theoretical amount of 0.1 M KOH in methanol was added in order to liberate the quinidine as the free base. After concentration to 1 ml, the solution was allowed to stand in the refrigerator overnight. Quinidine was removed by centrifuging and the supernatant transferred to a separate tube. Dry ether was then added when crystallisation of the potassium salt occurred. This was collected by filtration, washed with methanol–ether and dried. The latter was again converted to the quinidine salt as described above.

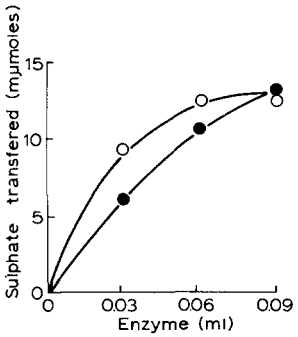
Material	*Spec. activity (counts/min per μmole)*
Quinidine estrone sulphate 1	216
Potassium estrone sulphate	191
Quinidine estrone sulphate 2	195

Yield of products derived from protein-bound acceptors

The enzyme isolated in the presence of mercaptoethanol yielded the products shown in Fig. 1 on incubation with [^{35}S]PAPS of high specific activity. An evaluation of the actual amounts of these radioactive products, formed by sulphation of bound acceptors, was then carried out by using pure [^{35}S]PAPS of known specific activity and measurement of the 'ester sulphate' fraction by the Wengle method[3]. Results are shown in Fig. 3. A near-linear response to enzyme concentration was obtained when incubation was limited to 2 h. Increasing the time of incubation to 6 h yielded 9 mμmoles of sulphate, as ester sulphate, per 200 μg of protein.

Estimated yield of estrone sulphate derived from enzymically bound estrone

An approximate estimate of the amount of estrone sulphate liberated was gained by carrying out a chromatographic separation of the radioactive ester sulphate. An experiment similar to that outlined in Fig. 3 was performed with the exception that the amount of added cold PAPS was reduced to 1.3 mμmoles. The enzyme employed (180 μg) was the same as used in Fig. 3 and incubation was carried out for 5 h. Aliquots were chromatographed on Whatman No. 1 paper in Solvent C and scanned with a Nuclear-Chicago Actigraph II instrument, Model 1032. SO_4^{2-} and PAPS had R_F's of 0.9 and 0.8, respectively. Radioactive ester sulphates had R_F values between 0.6 and 0.7 whereas the estrone sulphate had an R_F of 0.33. The sum total of sulphate transferred to endogenous acceptors was determined by weighing



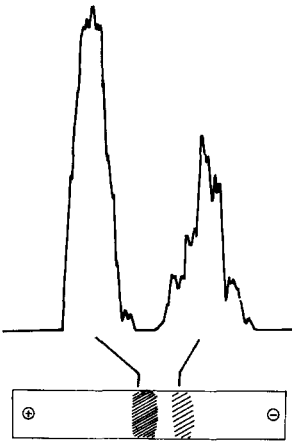


Fig. 3. Effect of increasing concentration of enzyme on the yield of radioactive products derived by incubation with [^{35}S]PAPS. The incubation contained 0.05 M Tris–HCl (pH 8.1), 12 mM Mg^{2+}, 15.6 mμmoles pure PAPS, [^{35}S]PAPS, $7.0 \cdot 10^4$ counts/min and the enzyme used in Fig. 1. Total vol. was 0.22 ml. Incubation was continued for 2 h (●—●) or 6 h (○—○) and after heating the ester [^{35}S]sulphate was determined by the Wengle method[8]. It was established that the enzyme itself was stable during the incubation since 0.09 ml of buffered enzyme, pre-incubated for 4 h prior to addition of [^{35}S]PAPS and then incubated for a further 2 h, gave 95% of the yield obtained in the normal incubation carried out for 6 h. Results are corrected for a zero time control which gave near-background counts.

Fig. 4. Separation and estimation of the major contaminating protein from the estrogen sulphotransferase preparation isolated in the presence of mercaptoethanol[1]. Paper electrophoresis of the enzyme used in Fig. 1 and Fig. 3 was carried out in 0.05 M barbiturate–HCl (pH 8.6). After staining with 1% Amido Black, the paper was treated with mineral oil and scanned with a Joyce Loebl Chromoscan Densitometer.

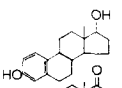
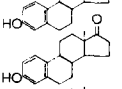
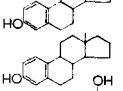
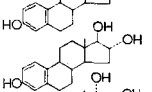
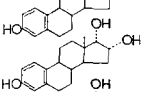
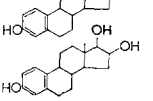
the areas under the curves and the percentage of this total corresponding to estrone sulphate was calculated. It was necessary to reduce the concentration of PAPS in this experiment in order to be able to register the estrone sulphate peak on the resulting scan of the chromatograph. Under conditions where the concentration of PAPS was non-limiting (to a first approximation), the total yield of ester sulphates was 9 mμmoles per 200 μg of protein (Fig. 3). If we assume for purposes of calculation that the percentage estrone sulphate in the 9 mμmole is the same as that estimated under conditions where the PAPS concentration was considerably reduced, then the yield of estrone sulphate was 0.33 mμmole per 200 μg of protein. The estrogen sulphotransferase content of the enzyme preparation was determined by paper electrophoresis followed by staining and scanning as shown in Fig. 4. It was previously demonstrated that the major protein was readily separated from the estrogen sulphotransferase by electrophoresis on Geon resin or acrylamide gel[1]. From Fig. 4, the major protein comprised 65% of the total; estrogen sulphotransferase would then represent the greater percentage of the remaining protein (see Fig. 7, Part IV[1]). A yield of 0.33 mμmole of estrone sulphate per 70 μg of estrogen sulphotransferase would represent 0.33 mole of estrone per mole of enzyme, based on a molecular weight of about 67 000 for the non-associated enzyme[1]. Much lower values (0.03 mole estrone per mole enzyme) were obtained when Fraction A_2 was incubated with 11 mμmoles of [^{35}S]PAPS under similar conditions to those given in Fig. 3. However this enzyme preparation had lost a high percentage of its activity on prolonged storage.

K_m and $v_{max.}$ values were determined for a number of estrogens in order to obtain data on possible structural requirements necessary for the estrogen to act as a substrate for the enzyme. The non-associated A form of the enzyme was used since uncomplicated linear Lineweaver–Burk plots were obtained[1]. Kinetic measurements were determined as described previously; estrogen being varied in the presence of fixed near-saturating levels of pure PAPS (refs. 1, 3). Results are shown in Table II.

TABLE II

K_m AND $v_{max.}$ VALUES FOR VARIOUS ESTROGENS

Conditions were essentially those described previously for the standard enzyme assay[1]. Estrogen concentrations in the range 70 to 8 μM were examined using Fraction A enzyme[3] which had been stored at −20° for 6 months. This particular preparation had been dialysed against 0.01 M Tris–HCl (pH 7.5) to remove phosphate and then frozen. Protein, 1.5 mg/ml.

Substrate	*Structure*	K_m *(μM)*	$v_{max.}$ *(mμmoles/20 min per 0.05 ml enzyme)*
17 β-Estradiol		14*	—*
Estrone		14	2.0
17-Deoxyestrone		14	1.3
Estriol		5	2.2
17-Epiestriol		8	1.6
16-Epiestriol		14	2.9

* K_m as determined previously[1,3]. The remaining estrogens were examined in the one experiment.

DISCUSSION

The demonstration of estrone bound to the purified enzyme could perhaps suggest that this particular estrogen is the true substrate of the enzyme. This interpretation does correlate with the contrasting behaviour of estrone as compared to 17 β-estradiol and estriol at high substrate concentrations in the velocity–substrate curves[1]. Since the fully associated enzyme exhibits allosteric properties, control mechanisms may possibly function as a result of feed-back effects of estrone metabolites.

On the basis of the 9 mμmoles of 'ester sulphate' per 200 μg of protein (Fig. 3), an average molecular weight of 22 000 would be required if we regard all the protein as sulphotransferase containing 1 mole of bound acceptor per mole of enzyme. On the other hand, the acceptors could possibly be adsorbed to enzyme, or foreign protein, and in some way still be capable of acting as substrates for sulphotransferase enzymes. Whatever the explanation, the acceptors are very firmly bound since

repassage of the enzyme, isolated in the presence of mercaptoethanol, through a second DEAE-cellulose column did not alter the pattern of labelled products shown in the chromatograms of Fig. 3. The possibility that the major component of these products may arise by sulphation of exogenous acceptors, introduced in buffers *etc.* cannot be ruled out. However a major radioactive zone, with very similar chromatographic properties to the component in question, was consistently present in incubations of other tissue extracts and [^{35}S]PAPS, carried out in phosphate buffer in place of the normal Tris–HCl (ref. 6). The determined level of bound estrone can only be regarded as providing some indication of the true value. A more direct approach using spectrofluorimetric assays will be carried out when larger amounts of highly purified enzyme can be prepared.

Measurement of the K_m values of a group of estrogens, as a means of obtaining data on structural requirements for binding to the enzyme, have yielded interesting results. The most significant finding was the ability of 17-deoxyestrone to act as a substrate and to possess a K_m identical to a number of other estrogens, lacking hydroxyl groups on the α-side, but containing such groups as β-substituents (Table II). An overall fit, involving multiple sites of interaction by forces of the Van der Waals type, is indicated by such results. 17-Deoxyestrone has also been found to bind strongly to β-hydroxysteroid dehydrogenase (EC 1.1.1.51) (ref. 7) and to act as a powerful inhibitor of the enzyme. In addition, certain steroids completely lacking oxygen functions on any carbon atom have been shown to possess biological activity, *e.g.* androstane shows androgenic activity[8]. Such studies implicate the importance of the steroid nucleus in the binding to protein or receptor site.

As mentioned above, introduction of β-hydroxyl groups did not alter the K_m value, although effects on the $v_{max.}$ were observed. These results, in turn, can best be interpreted by the assumption that the enzyme reacts with the rear or α-face of the estrogen. Evidence for the binding of proteins to the α-face of steroids has been documented by WESTPHAL[2]. It is of interest that the binding of estrogens to human placental estradiol 17 β-dehydrogenase (EC 1.1.1.i) has been suggested[9] to be *via* the rear side and the K_m values for estrone and 17 β-estradiol were reported as 20 μM; values very close to those recorded here (Table II). Introduction of α-hydroxyl groups into ring D of the estrogen leads to lowered K_m values with estrogen sulphotransferase, which could possibly implicate such groups as forming additional polar bonds with appropriate receptors on the enzyme surface.

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