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ENZYMIC SYNTHESIS OF STEROID SULPHATES

IV. THE NATURE OF THE TWO FORMS OF ESTROGEN SULPHOTRANSFERASE OF BOVINE ADRENALS

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SUMMARY

1. The nature of the two forms (A and B) of estrogen sulphotransferase (3'-phosphoadenylylsulphate:estrone sulphotransferase, EC 2.8.2.4) isolated by DEAE-cellulose chromatography, has been investigated. In contrast to the A form, the B form gave non-linear double-reciprocal kinetic plots. Similar plots were obtained when cysteine was added to the A form.

2. Form B was shown to be converted to Form A on standing.

3. Only the B form was obtained when the enzyme was isolated in the presence of mercaptoethanol.

4. Form B was shown to be related to A as trimer (or perhaps tetramer) to monomer, by thin-layer chromatography on Sephadex G-200. The molecular weight of the monomer was estimated to be about 67 000 by this method.

5. The ability to associate to the trimer is suggested to be related to the possession of a conformation dependent on the maintenance of a SH group, or groups, in the reduced state. Non-linear kinetic curves are explained by interaction of binding sites on the associated protein.

6. Allosteric properties exhibited by the fully-associated enzyme are discussed in terms of control mechanisms which may operate at the level of steroid sulphotransferase in endocrine organs.

7. Both forms showed three to four closely migrating protein bands on acrylamide gel electrophoresis which persisted throughout the purification procedures. Evidence was presented that these bands represented individual isoenzymes.

8. The amino acid composition of the highly purified enzyme has been determined.

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

INTRODUCTION

In the previous paper (Part III)[1], the isolation of estrogen sulphotransferase (3'-phosphoadenylylsulphate:estrone sulphotransferase, EC 2.8.2.4), free of other contaminating sulphotransferases, was described. Two forms of the enzyme, A and B, were obtained by chromatography on DEAE-cellulose, one of which (Form A) was more stable than the other. The general properties of the enzyme were examined using the A form in all experiments. Cysteine was observed to activate this form of the enzyme and an examination of this activation by kinetic means revealed unusual velocity–substrate curves. Form B alone, *i.e.* in the absence of added cysteine, produced similar curves. Purified samples of A and B enzyme have now been prepared and their physical properties examined. Form B has been found to be an associated protein, being related to A as trimer (or perhaps tetramer) to monomer. Interaction of binding sites on the associated form is believed to explain the abnormal kinetic behaviour.

MATERIALS

In general materials used were as described in the preceding paper[1]. Pure proteins, used for calibration of thin-layer plates of Sephadex G-200 were obtained from the following sources: cytochrome *c*, Nutritional Biochemicals; hemoglobin, twice recrystallised, Sigma Chemical; lysozyme, Eastman Kodak; trypsin, A grade and bovine serum albumin, A grade, California Corp. for Biochemical Research; bovine γ-globulins (Cohn fraction No. II-1,2) Commonwealth Serum Laboratory (Melbourne). Carbowax 20M was obtained from Union Carbide (Australia), and Geon 426 resin from B.F. Goodrich.

METHODS

Kinetic experiments

Incubations were carried out for 20 min, using the conditions described in the standard assay procedure in the previous paper[1]. 0.26 M stock solutions of cysteine HCl (Sigma) were prepared and neutralised with NaOH immediately prior to addition to the incubations.

Isolation and purification of A and B forms of the enzyme

DEAE-cellulose chromatography, employing a concave gradient was used to separate the two forms of the enzyme[1]. Phosphate was removed from the pooled Fractions A and B by dialysis, and a second $(NH_4)_2SO_4$ fractionation was carried out. The most active fraction of A was obtained at 0.55–0.62 satn. (Fraction A_2) and the corresponding fraction of B was obtained at 0.55–0.58 satn. (B_2). Table I summarises the purification steps.

The four fractions obtained after the second $(NH_4)_2SO_4$ fractionation were separately dialysed against 0.01 M Tris–HCl buffer (pH 7.5). Fractions A_2 and B_2 were lyophilised and stored at −20°. Fractions A_3 and B_3 were stored in ice.

Isolation of the enzyme in the presence of mercaptoethanol

600 g of bovine adrenal glands were homogenised and the enzyme isolated

exactly as previously described[1], with the exception that 2-mercaptoethanol, at a final concn. of 3 mM, was added to the extracting buffer and to all the solutions (including the DEAE-cellulose eluting buffers) used subsequently in the enzyme purification. The 0.55–0.80 satd. $(NH_4)_2SO_4$ fraction was chromatographed on a 4 cm × 30 cm column of DEAE-cellulose using concave gradient conditions. A single peak of enzyme activity was obtained in this case (Fig. 5). The active fractions were pooled, dialysed to remove phosphate and passed through a second column of DEAE-cellulose of the same dimensions and employing mercaptoethanol in the buffers. Again only a single peak of enzyme activity was found. This peak was dialysed and concentrated against 20% (w/v) carbowax 20M (polyethylene glycol 20 000) in 0.01 M Tris–HCl, 1 mM EDTA and 3 mM 2-mercaptoethanol. Gel electrophoresis revealed the presence of a major protein contaminant (Fig. 7) which was removed in the next stage of the purification.

Electrophoresis on Geon resin

The major protein contaminant was removed by taking advantage of its high electrophoretic mobility. Geon 426 (polyvinyl chloride) was stirred for 2 h with excess 2 M HCl. The resin was then washed with water on a Buchner funnel until the pH of the washings was neutral and absorbance at 280 mμ was negligible. It was then suspended in a minimum volume of 0.05 M barbital–HCl buffer (pH 8.6) containing 3 mM 2-mercaptoethanol. The thick suspension was poured onto the tray of an LKB electrophoresis apparatus after removal of the supporting plates. A layer 28 cm × 18 cm and approx. 3 mm thick was formed from which excess buffer was removed by filter paper wicks at both ends of the tray. These then served as contacts for the same buffer introduced into the reservoir tanks. Current was passed through the resin for 30 min at 350 V. A trough (12 cm × 0.5 cm × 0.3 cm) was then cut across the resin about 8 cm from the cathode end. 2 ml (100 mg of protein) samples of the above enzyme solution were used for each run. Dry Geon 426 was added to the enzyme solution to form a heavy suspension and this was poured evenly into the trough. The tray was mounted onto the tanks and allowed to equilibrate for 15 min. Current was passed for 10 h at 200 V, the voltage then being increased to 340 V and the electrophoresis continued for a further 4 h. All operations were carried out at 5°. The tray was then removed from the assembly, the resin cut into 1-cm sections and each section transferred to 15 ml centrifuge tubes. Protein was eluted by stirring with 5 ml of 0.1 M Tris–HCl buffer (pH 8.1) containing 3 mM mercaptoethanol. After centrifuging, absorbance measurements were made and estrogen sulphotransferase activity assayed on 0.1-ml samples (Fig. 8). Active fractions were pooled and concentrated against carbowax.

Attempted crystallisation, by addition of satd. $(NH_4)_2SO_4$ to faint turbidity, was unsuccessful. The bulk of the protein was then precipitated by saturating the solution with $(NH_4)_2SO_4$. The precipitate was collected and dissolved in a small volume of 0.05 M Tris–HCl (pH 7.5) containing 3 mM mercaptoethanol (Fraction B-SH).

Amino acid analysis

In order to obtain a highly purified sample of the enzyme suitable for amino acid analysis, 6 electrophoretic runs on acrylamide gel were carried out employing

8 tubes per run and 0.05 ml of the above enzyme solution per tube. Sections 1–2 cm from the start of the small-pore gel (see Fig. 9) were cut out, homogenised with Tris buffer, shaken for 1 h and then centrifuged. The supernatant was dialysed overnight against the same buffer and concentrated against 20% (w/v) carbowax. It was then transferred to a heavy-walled tube and after lyophilisation, the residual protein was hydrolysed at 110° for 20 h (ref. 2). HCl was removed by evacuation in a desiccator containing NaOH. The residue was dissolved in buffer and aliquots were analysed on a Beckman amino acid analyser Model 120/C, without preliminary exposure to air to oxidise cysteine to cystine[2]. Analyses were kindly performed by Mr. M. SMITH and Miss JOAN BACK of the Division of Food Preservation, CSIRO.

Thin-layer chromatography on Sephadex G-200

The technique of MORRIS[3] was employed which gave a linear plot of R_{Hb} (ratio of distance travelled by a protein to that travelled by hemoglobin) against the logarithm of the molecular weights of proteins. A superfine grade of Sephadex G-200 was used, and after preparing and equilibrating the plates, 1 μl of the test solution, containing 1–20 μg of protein, was applied. Runs of 4–5 h duration were employed, in which time the hemoglobin had moved about 70 mm. Zones were located by the technique of overlaying the plate with a 20 cm × 20 cm sheet of Whatman 3 MM paper and drying in an oven at 80° for 30 min. Positions of the initial spots were marked on the paper and the protein zones were located by staining with 1% (w/v) Naphthalene Black 10B in 7% (v/v) acetic acid.

RESULTS

Kinetics of Form A in the presence of cysteine

When initial velocity measurements were carried out with Form A enzyme in the presence of cysteine, utilising changing estrogen at fixed adenosine-3′-phosphate-5′-phosphosulphate (PAPS) concentrations, an immediate rise in the slope of the velocity–substrate curve was evident. The Lineweaver–Burk plots became complex curves (Fig. 1), which was in marked contrast to the linear functions realised with Form A enzyme alone[1]. The complexity of the double reciprocal plots obtained in the presence of cysteine was not relieved by addition of Mg^{2+} (Fig. 1). In Fig. 2, the effect of cysteine addition is perhaps more clearly seen: initial velocities being compared in the absence and presence of this compound *plus* Mg^{2+}.

Effects of cysteine on the A enzyme, at changing PAPS levels and constant estrogen concentration, is shown in Fig. 3. Again non-linear double reciprocal plots are obtained. Addition of Mg^{2+} is observed to cause a marked increase in reaction rate.

Kinetics of Form B in the absence of cysteine

When Fraction B enzyme was examined in the absence of cysteine, it behaved in a similar manner to Fraction A *plus* cysteine (Fig. 4). This suggested that the A form of the enzyme could be converted into the B form by addition of SH groups; a process which possibly involved reduction of disulphide linkages in the A enzyme.

Isolation of enzyme in the presence of mercaptoethanol

When the bovine glands were extracted in the presence of 3 mM 2-mercapto-

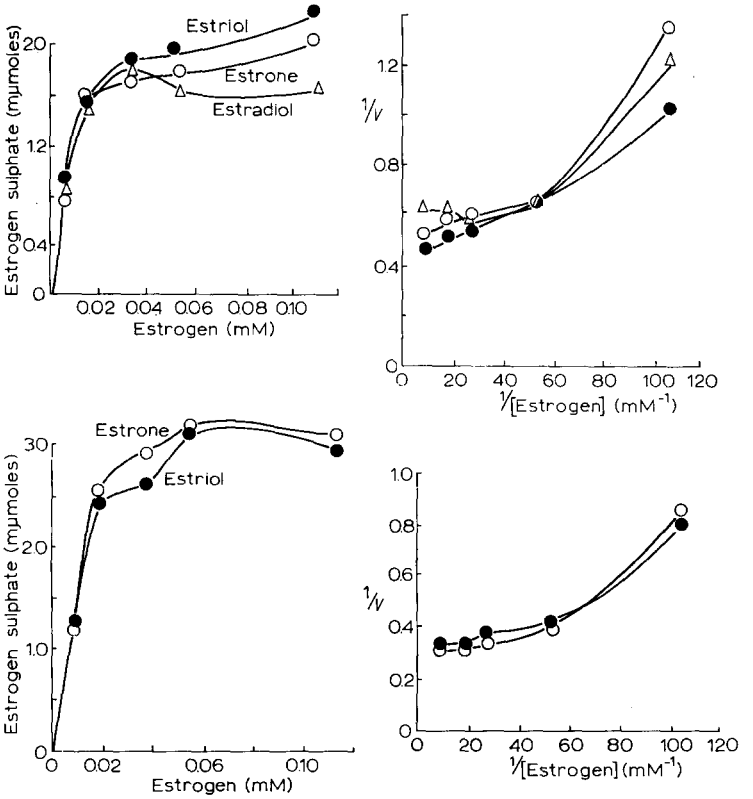


Fig. 1. Effect of cysteine on kinetics employing Form A enzyme. Top, standard enzyme assay in the absence of Mg^{2+}; bottom, Mg^{2+}, 20 mM. The cysteine concn. was 10 mM in both cases. Enzyme: lyophilised Fraction A (200 μg protein).

ethanol, and when this concentration of the thiol was maintained during all subsequent procedures, then only one peak of activity appeared during the DEAE-cellulose chromatography. This peak was eluted in the B area (Fig. 5). Form A could then occur as a result of an oxidation of Form B, although its separate occurrence and

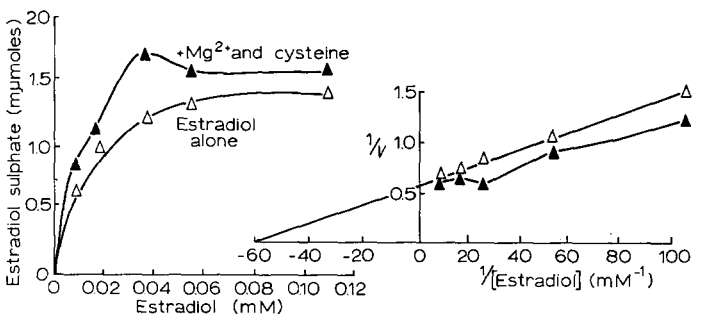


Fig. 2. Kinetics using Form A enzyme in the absence and presence of cysteine *plus* Mg^{2+}. Concentrations were the same as in Fig. 1.

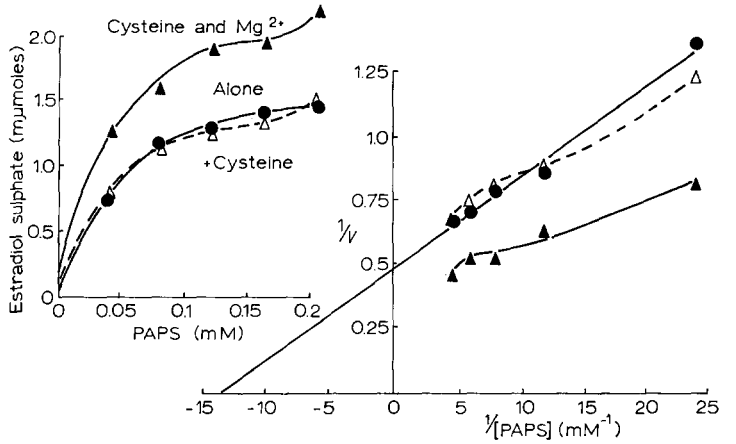


Fig. 3. Effect of varying PAPS, at fixed estrogen concn., using Form A enzyme in the presence and absence of cysteine, and cysteine *plus* Mg^{2+}. 17 β-Estradiol concn., 0.065 mM. Enzyme: lyophilised Fraction A (200 μg protein).

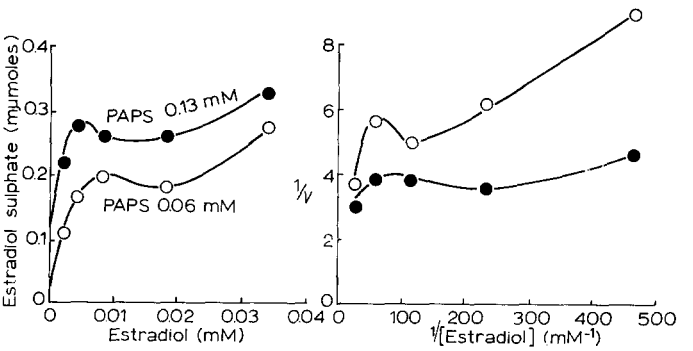


Fig. 4. Effect of varying estrogen at two fixed levels of PAPS with Form B enzyme. The latter was obtained by DEAE-cellulose chromatography and was eluted after Form A enzyme[1]. Volume of enzyme (stored in the frozen state), 0.04 ml (144 μg protein). Cysteine has not been added to these incubations.

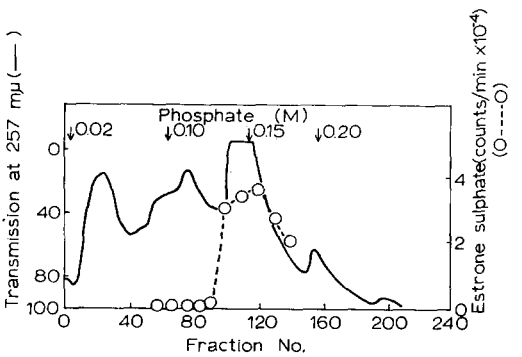


Fig. 5. DEAE-cellulose chromatography of the 0.5–50.80 satd. $(NH_4)_2SO_4$ fraction of enzyme isolated in the presence of mercaptoethanol. Concave gradient elution was carried out as previously described[1].

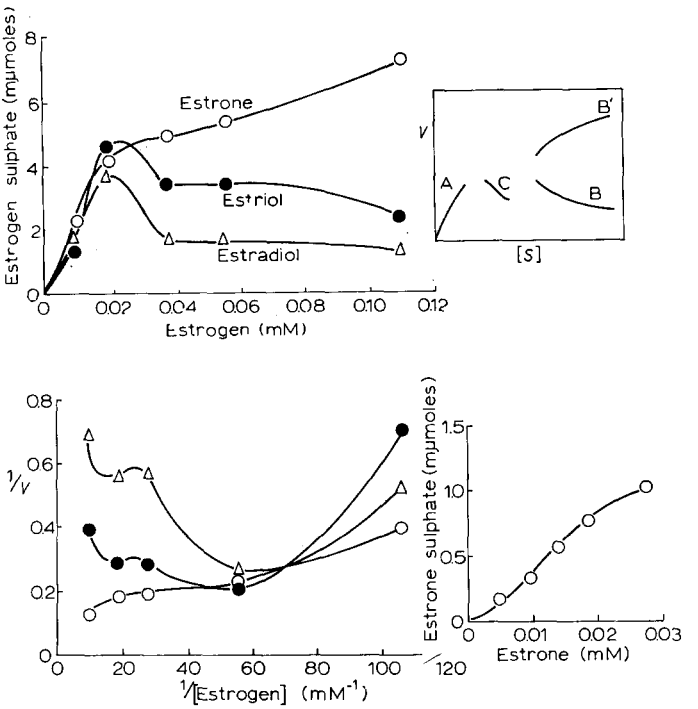


Fig. 6. Kinetics with the enzyme isolated in the presence of mercaptoethanol (see Fig. 5). Top: left, initial velocities with variable estrogen; right, general shape of an initial velocity–substrate curve for an enzyme exhibiting interaction between multiple binding sites (REINER, ref. 8). Bottom: left, double reciprocal plots of data presented on top, left; right, sigmoid-shaped curve revealed by measurements at low estrogen concentrations. The enzyme was stored in ice under N_2, for 5 days for the data presented in the top of the figure, and for 6 months for the data shown on the right of the lower portion. A vol. of 0.03 ml was employed (198 μg protein).

TABLE I

ISOLATION OF PURIFIED SAMPLES OF A AND B FORMS OF THE ENZYME

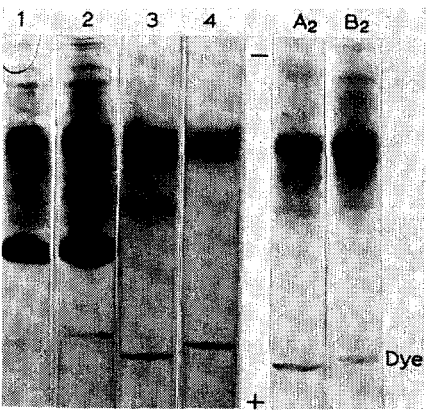
Wt. of bovine adrenal glands: 1025 g. DEAE-cellulose column: 6 cm × 40 cm, concave gradient elution being employed[1].

Treatment	*Protein (mg)*	*Spec. activity (mμmoles estrone sulphate/mg per 20 min)*	*Units*
1st $(NH_4)_2SO_4$(0.55–0.80satn.)	5400	7.6	41 000
1st DEAE-cellulose			
A form	463	23.1	10 700
B form	245	54.0	13 200
2nd $(NH_4)_2SO_4$			
A_2 (0.55–0.62 satn.)	5.9	97.0	572
A_3 (0.62–0.73 satn.)	55.5	39.2	2180
B_2 (0.55–0.58 satn.)	28.0	95.0	2660
B_3 (0.58–0.68 satn.)	57.3	52.3	3000

perhaps biological function in the tissue may be real. The kinetic behaviour of this preparation is shown in Fig. 6 and the similarity in the shape of the curves to other preparations of Form B (Fig. 4) and to Form A *plus* cysteine (Fig. 1) is apparent.

Physical properties

Preparation of purified samples of A and B. In Table I the preparation of samples of the A and B forms having high specific activities is outlined. The behaviour of Fractions A_2 and B_2 on gel electrophoresis are compared in Fig. 7. An additional specimen of the B form, which was used for molecular weight determinations,



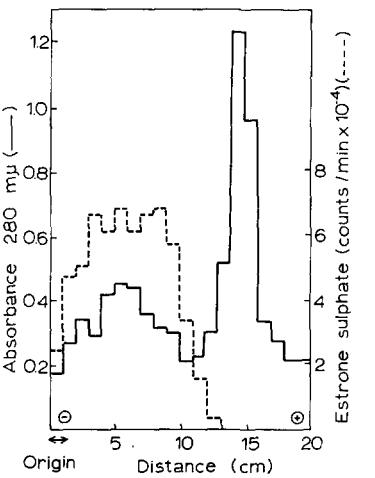


Fig. 7. Gel electrophoresis of enzyme fractions. 1 and 2, enzyme isolated after one and two passages through DEAE-cellulose (see Fig. 5); 3, enzyme, as shown in 2, after purification by electrophoresis on Geon resin (Fraction B-SH); 4, enzyme, as shown in 3, after purification by acrylamide-gel electrophoresis. A_2 and B_2 (see Table I) were run separately to 1–4.

Fig. 8. Purification of the enzyme by electrophoresis on Geon resin. The enzyme used was obtained as shown in Fig. 5.

was prepared from tissue extracted in the presence of mercaptoethanol. The enzyme fraction from the DEAE-cellulose column was again passaged through a similar column and then subjected to further purification by electrophoresis on Geon 426 resin (Fig. 8). Stages of purification are revealed by the gel-electrophoretic patterns (Fig. 7). Highly purified enzyme, suitable for amino acid analysis, was obtained by gel electrophoresis in a number of separate runs. The purity of this final sample is also shown in Fig. 7 and results of the amino acid analysis is given in Table II.

Conversion of Form B to Form A. It was mentioned in the previous paper[1] that Form B lost activity at a much greater rate than Form A when the two separated enzymes were isolated from the DEAE-cellulose columns. Conversion of Form B to Form A was observed to take place as shown in Fig. 10. Fraction B_3 (Table I), which had been stored in ice for 8 days, was observed to yield both A and B forms when repassed through DEAE-cellulose. The possibility that A form may have been a contaminant carried over into Fraction B can be ruled out since the elution pattern revealed that A and B were obtained in approx. equal amounts.

TABLE II

AMINO ACID COMPOSITION OF A HIGHLY PURIFIED B FORM OF THE ENZYME

Corrections have not been made for destruction during acid hydrolysis[2]. The enzyme was isolated in the presence of 3 mM mercaptoethanol and purified as described in METHODS. Fig. 7 shows its behaviour on gel electrophoresis.

Amino acid	*Moles per cent of total amino acids*	*Amino acid*	*Moles per cent of total amino acids*
Lys	8.8	Ala	9.1
His	2.4	Cys	Present*
Arg	3.2	Val	6.0
Asp	13.8	Met	1.3
Thr	5.0	Ileu	2.9
Ser	6.7	Leu	8.8
Glu	9.7	Tyr	2.4
Pro	5.7	Phe	5.8
Gly	8.4		

* Peak too small for accurate measurement.

Molecular weight determinations. Because of the comparatively small amounts of purified enzyme available the newly developed technique[3,4] of molecular weight determination by thin-layer chromatography on Sephadex G-200 was selected to investigate the biophysical relationship between the A and B forms of the enzyme. Results are summarised in Table III. Fraction A_2 and the B-SH fraction, obtained by elution from the Geon-resin electrophoresis, were used for these determinations. The accuracy of the above method is claimed to be of the order of 20% (ref. 4). This was about the level of reproducibility obtained here, and would relate the B form to the A form, as trimer to monomer although the possibility of B being a tetramer cannot be ruled out. Support for the trimer to monomer relationship has been obtained in preliminary experiments using columns of Sephadex G-200 as described by ANDREWS[4].

Isozyme patterns. It became apparent in the early stages of this investigation that a closely migrating group of 3 or 4 protein bands was always present in extracts containing the enzyme upon examination by gel electrophoresis. Furthermore, as the enzyme was purified, the same pattern of zones, travelling in a predictable position on the gels, was always obtained (Fig. 7). That the enzyme was associated

TABLE III

MOLECULAR WEIGHT DETERMINATIONS OF A AND B FORMS CARRIED OUT BY THIN-LAYER CHROMATOGRAPHY ON SEPHADEX G-200

Enzyme form	*Fraction*	R_{Hb}*	*Estimated mol. wt.*
A	A_2	1.13**	67 000
B	B-SH	1.44***	191 000

* Migration distance relative to hemoglobin.
** Mean of 5 separate determinations.
*** Mean of 4 separate determinations.

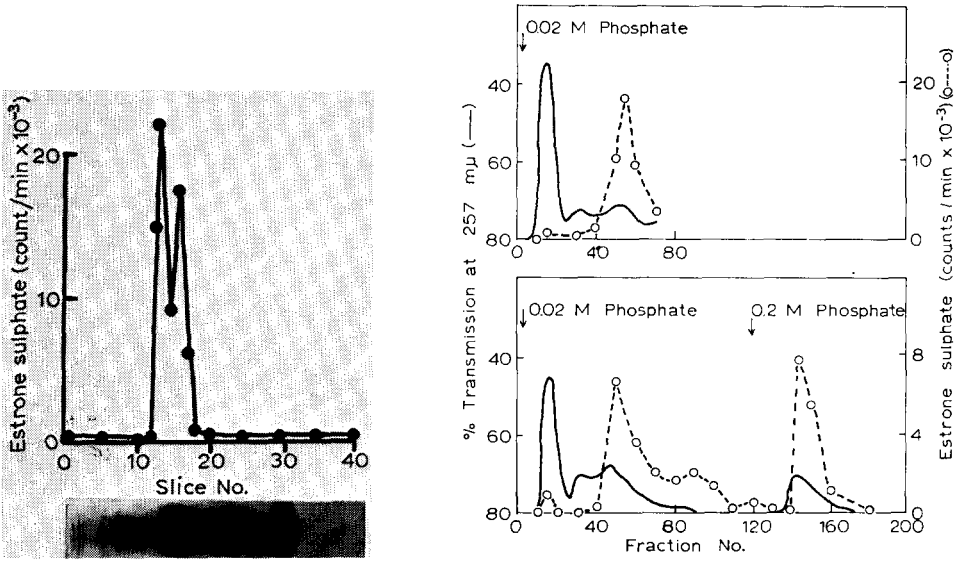


Fig. 9. Enzyme distribution in the protein separated by acrylamide-gel electrophoresis. Enzyme isolated in the presence of mercaptoethanol was employed (Fig. 5). A duplicate unstained gel was placed in a rack fitted with a screw device such that one turn moved the gel 1 mm from the opposite end of the rack. This section was cut with a razor blade and enzyme activity measured after freezing and thawing the gel in 0.1 ml of 0.1 M Tris–HCl (pH 8.1).

Fig. 10. Conversion of Form B enzyme to Form A as revealed by chromatography on DEAE-cellulose. The top portion shows the passage of Fraction A_3, and the lower curve the passage of Fraction B_3 (see Table I), through a column (1 cm × 30 cm) of DEAE-cellulose employing concave gradient elution as in Fig. 5. The volume of the fractions was 2 ml. 55-mg samples of the enzyme fractions, which had stood in ice for 8 days, were applied to the column in each case.

with this closely migrating group of proteins is strongly suggested by the results shown in Fig. 9. Enzyme activity was present throughout this band and was not associated with any other protein as shown by cutting the gel into 1-mm segments and carrying out enzyme assays. The break in the curve relating enzyme activity to slice number would also indicate that enzyme activity is associated with more than one individual protein component of the complex—hence an isozyme pattern is indicated. Despite much effort, it has not been possible to locate the enzyme on the gel by employing an *in situ* staining reaction as is done with isozymes such as lactate dehydrogenase.

DISCUSSION

The complex nature of the double-reciprocal plots obtained with Form A enzyme in the presence of cysteine suggested that: (i) a second enzyme species with a lower K_m for estrogen was formed so that two separate enzyme forms co-existed. Two forms of hexokinase having widely differing K_m values have been shown to occur in human liver cells. Lineweaver–Burk plots obtained with the crude hexokinase gave points compounded of two linear functions and extrapolation of the slopes yielded the two apparent K_m values[5]. (ii) The enzyme associated to produce

a new species with multiple and interacting binding sites such that S^2, S^3 . . . or S^n terms, where S equals substrate, would appear in the denominator in the reciprocal form of the rate equation, leading to curved double reciprocal plots.

The possibility (i) can be excluded since curves, rather than compounded linear double reciprocal plots, were obtained and the B form of estrogen sulphotransferase appears to be a discreet species as shown by DEAE-cellulose chromatography and thin-layer chromatography on Sephadex G-200. Enzymes with multiple substrate binding sites which do not interact behave kinetically as a mixture of enzymes with single binding sites[6]. Interaction between the sites, as outlined in (ii) above, is exemplified in the allosteric enzymes: such interaction in this case usually showing enhancement of substrate binding leading to the now familiar sigmoid-shaped curve relating initial velocity, v, to substrate concentration, S (ref. 7).

REINER[8] has considered the case of an enzyme with n interacting binding sites such that

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_1;\ C_1 \xrightarrow{k_{1a}} E + P$$

$$S + C_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C_2;\ C_2 \xrightarrow{k_{2a}} C_1 + P \text{ etc.},$$

where E equals enzyme, S substrate and P product. A rate equation for the general case of n interacting sites was derived and a partial analysis for extreme and intermediate values of S led to a generalised form of the v–S curve. This curve is reproduced in Fig. 6. When $S = 0$, $v = 0$ and v approaches $k_{na}Et$ as S becomes very large. Segments B and B′ are two possibilities for the terminal part of the curve. Their slopes are either positive or negative depending on the value of terms containing the intrinsic constants k_{na} and $k_{n-1,a}$ in the derived function dv/dS. If the situation is that of Segment B, there must be a peak somewhere between Segments A and B. Segment C is a region of possible negative slope for moderate values of S.

When the general shape of the theoretical curve is compared to the curve obtained with the fully associated estrogen sulphotransferase, the agreement is quite close (Fig. 6). Thus in the case of both 17 β-estradiol and estriol, a negative slope at high substrate concentrations, corresponding to Segment B above, is accompanied by a peak ($dv/dS = 0$) at lower substrate concentrations. Estrone gives a positive slope at high concentration, corresponding to Segment B′, which is unaccompanied by a peak value at lower substrate concentrations. The apparent allosteric behaviour, indicated by the sigmoid-shaped curve at low substrate concentrations, will be discussed later. Kinetic evidence that the B enzyme was an associated structure was supported by the molecular weight determinations. The more rapid loss of activity of the B, as compared to the A form after isolation from DEAE-cellulose[1], could be explained by its conversion to the A form. This was shown to be the case by the rechromatography of Fraction B_3 (Fig. 10). The effect of cysteine on the A form was seen to produce similar kinetic curves to the B form alone. Slow oxidation of SH groups was perhaps indicated and substantiated by the isolation of the B form, unaccompanied by the A form, when enzyme was prepared in the presence of mercaptoethanol (Fig. 5). Thus the ability to associate to the trimeric state would seem to be dependent on the maintenance of a SH group, or groups, in the fully

reduced state. Oxidation of this group(s) evidently prevents the protein from adopting the conformation which is stabilised by association. Due to the limited amount of highly purified enzyme available, the amino acid analysis (Table II) can be regarded as providing information of a preliminary nature only. For this reason an accurate estimation of cysteine/cystine could not be carried our but it would appear likely that not more than 1 or 2 cysteine residues are present per monomeric unit. The SH content of the A and B forms will be determined when methods of obtaining larger amounts of purified samples can be developed.

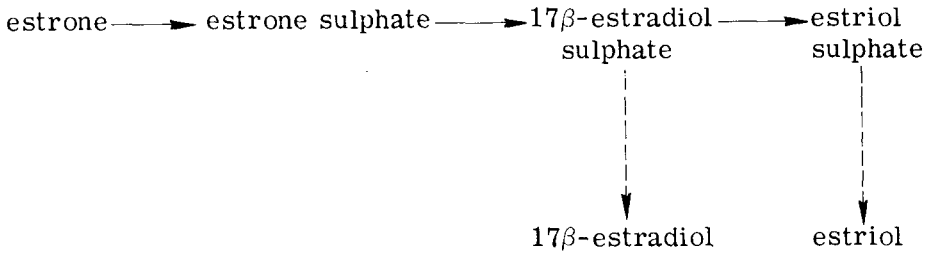
An alternative explanation of the kinetic behaviour exhibited by the B form could be related to the presence of the A form in such preparations. This could arise by oxidation, as discussed, or possibly by a normal equilibrium between the two forms. The appearance of an enzyme form with a higher K_m value for estradiol, and approximating that of the A form, could be construed from Fig. 4. High concentrations of steroid could also possibly favour dissociation of the B form, resulting in the type of curves obtained experimentally. This latter point is being checked by density-gradient centrifugation methods.

Although association of protein subunits to form tetramers is very common[9], the probable formation of trimers of aldolase (EC 4.1.2.b) and glutamate dehydrogenase (EC 1.4.1.2) (ref. 10) has been reported. The physical properties of the estrogen sulphokinase bear some similarities to phosphorylase *a* (EC 2.4.1.1). The latter is converted by *p*-chloromercuribenzoate to a new species having a molecular weight of 135 000 as compared to 495 000 for the native enzyme[11]. Upon treatment with cysteine the low molecular weight derivative is once again converted to phosphorylase *a*. These two species are related as monomer to tetramer and it would appear that the enzyme possessing fully-reduced SH groups exists in the associated form. On the other hand addition of excess SH groups, in the form of cysteine or mercaptoethanol, is known to cause reduction of disulphide linkages in some enzymes with formation of enzymically-active units of lower molecular weight[12].

Both forms of the enzyme produce apparently identical isozyme patterns when subjected to electrophoresis on acrylamide gel (Fig. 7). That such isozymes exist is highly probable as shown by the data in Figs. 7 and 9, which has been discussed previously. The association to the trimeric state is evidently reversed under the conditions employed in the acrylamide-gel electrophoresis—perhaps by oxidation of a sensitive SH group(s) or possibly by changes in other factors also necessary to maintain the associated structure.

The variance in behaviour of estrone as compared to 17 β-estradiol and estriol, obtained with the B enzyme (Fig. 6), is worthy of comment. Although this variance was not exhibited by form A enzyme in the presence of cysteine, this may have been due to incomplete conversion to the B form. Estrone was isolated from bovine adrenals in 1939 (see ref. 13) and the ability of the gland to sulphate estrone *in vitro* was demonstrated in 1963 by SNEDDON AND MARRIAN[14]. Homotropic effects exhibited by the associated form of estrogen sulphotransferase (Fig. 6) could mean that this enzyme falls into the allosteric class[7]. The possibility that arylamine sulphotransferase (EC 2.8.2.3) is an allosteric enzyme has been discussed by ROY[15]. In the case of estrogen sulphotransferase, the increased rate of sulphation of estrone at high substrate concentrations, as compared to the lowered rate with 17 β-estradiol and estriol, could perhaps suggest a specific role for estrone. One interpretation could be that

estrone is the true substrate for the enzyme. Conversion to the other estrogens might possibly occur after formation of the estrone sulphate:



Sulphate esters are known to be involved in certain steroid transformations (see INTRODUCTION, Part III[1]). Alternatively estrone sulphate may be secreted directly into the blood stream and serve as the transport form of the hormone.

In Part V it will be shown that estrone occurs bound to the purified estrogen sulphotransferase; thus lending support to the possibility that estrone may function as the normal physiological substrate for the enzyme.

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