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ENZYMIC SYNTHESIS OF STEROID SULPHATES

VII. ASSOCIATION–DISSOCIATION EQUILIBRIA IN THE STEROID ALCOHOL SULPHOTRANSFERASE OF HUMAN ADRENAL GLAND EXTRACTS

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SUMMARY

1. The enzymic formation of dehydroepiandrosterone sulphate (DHEAS) from dehydroepiandrosterone (DHEA) and 3′-phosphoadenylylsulphate (PAPS), using the high speed supernatant from human adrenal glands, was found to exhibit unusual kinetic properties; multipeaks being observed in the velocity–substrate plots.

2. A number of active enzyme species having different molecular weights were demonstrated by gel filtration and sucrose density-gradient centrifugation. The latter technique indicated the presence of monomer (mol.wt. 65 000), dimer, trimer and possibly higher associated states which existed in a slowly reversible association–dissociation equilibrium. Isolation of the individual species by density-gradient centrifugation was possible and changes in activity which accompanied the slow return to equilibrium showed that such activity was related to the state of association.

3. Both substrates modified the position of equilibrium as shown by density-gradient centrifugation; PAPS in high concentration (0.15 mM) caused complete dissociation to monomer while DHEA favoured association.

4. Mg^{2+} and cysteine, both of which activated the enzyme in kinetic studies, were shown to favour association at the same concentrations by the centrifugation technique.

5. The kinetic properties of the enzyme can be explained by the effects of substrate concentration on the position of equilibrium of associated structures which themselves possess varying catalytic activities. In addition to substrates, other environmental factors such as metal ion concentration and oxidation–reduction potential influenced the activity of the system, and their effects are discussed in terms of a possible control operating by such changes in the equilibrium of the association–dissociation reaction.

Abbreviations: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; PAPS, 3′-phosphoadenylylsulphate; PCMB, *p*-chloromercuribenzoate.

INTRODUCTION

Steroid sulphates have recently come into focus as possible intermediates in steroid biosynthesis and metabolism[1,2]. Present interest has arisen, in particular, from the discovery of high concentrations of cholesteryl sulphate and dehydroepiandrosterone sulphate (DHEAS) in the blood, the demonstration of the secretion of DHEAS by the human adrenal[2], and the further metabolism of steroid sulphates in reactions whereby the sulphate group remains intact[1]. The sulphates of 3β-hydroxy-Δ^5-steroids secreted by the foetal adrenals in pregnancy, are now known to be the major sources of placental estrogens[3]. However the significance of steroid sulphates in the normal state remains obscure at the present time.

Recent papers in this series[4-6] have described some interesting features of a purified estrogen sulphotransferase (EC 2.8.2.4) derived from bovine adrenals. These features have prompted us to examine the sulphation of steroid alcohols in the human adrenal. Dehydroepiandrosterone (DHEA) was chosen as substrate since it was sulphated at a greater rate than other steroids previously tested with crude extracts of human glands[7]. Other 3β-hydroxy-Δ^5-steroids, such as pregnenolone, 17α-hydroxypregnenolone and possibly cholesterol, could also be likely substrates for the enzyme. These compounds occupy a central position in pathways leading to androgens, estrogens and corticosteroids in the adrenal gland. The sulphation reaction might then be of importance in controlling directions or rates of subsequent steps.

The steroid alcohol sulphotransferase has in fact been shown to possess most unusual properties. It exhibits complex kinetics which apparently reflect a system in which a number of active forms co-exist in proportions which are dependent on concentration of substrates and environmental factors. Enzyme forms of varying activity correspond to a series of states of association of a monomeric unit*, present in a reversible association–dissociation equilibrium. These properties suggest that the sulphotransferase could function as a control point in steroid metabolism.

MATERIALS

Steroids

DHEA was obtained from Sigma Chemical. Pregnenolone was a gift from Dr. R. LACK. Cholesterol (British Drug Houses, Lab. reagent) was recrystallised twice from ethanol. DHEAS was synthesised as described previously[7] and cholesteryl sulphate was a gift from Dr. A. B. ROY. Other steroids were obtained commercially.

Labelled compounds, nucleotides, and other reagents, were as described previously[4]. Solutions of ascorbic acid and cysteine hydrochloride were prepared freshly each day and adjusted to pH 7.0 with NaOH. Ox-liver catalase (EC 1.11.1.6) (3000 Units/mg) was purchased from the Nutritional Biochemical Corp.

METHODS

Paper chromatography

Whatman No. 1 paper was employed in descending chromatography in a paper-lined tank. Solvent A: di-isopropylether–*tert.*-butanol–conc. ammonia–water (6:4:1:9, by vol.). This was prepared freshly each day.

* Monomeric unit refers to the lowest molecular weight species showing enzyme activity.

Radioactivity measurements

Strip chromatograms were scanned with a Nuclear Chicago Actigraph (Model 1036) instrument, or where specific activity was low, chromatograms were cut into segments and counted by liquid scintillation. A Nuclear Chicago Unilux (Model 6850) instrument was used for liquid scintillation counting and the method, as applied to enzyme assays, was a previously described[4].

Preparation of adrenal high speed supernatants

Human female adrenal glands, obtained from breast cancer patients undergoing adrenalectomy, were stored frozen. Glands stored for periods up to 1 year have been used in this study. The tissue was homogenised in the cold with 3 vols. of 0.08 M KCl in 0.05 M Tris–HCl (pH 7.5) using an Ultra-Turrax homogeniser[8]. The homogenate was centrifuged at 20 000 × *g* for 20 min, lipid material removed and the supernatant respun at 100 000 × *g* for 1 h. The supernatant, containing 20–30 mg protein per ml, was divided into 1-ml aliquots and stored at −20°.

Enzyme assay

The method was very similar to that used for assaying estrogen sulphotransferase[4], which was based on the method of WENGLE[9]. Unless otherwise stated incubations contained 0.05 M Tris–HCl buffer (pH 7.5), 10 mM Mg^{2+}, [^{35}S] 3′-phosphoadenylylsulphate (PAPS) (100 000 counts/min), DHEA in 0.005 ml propylene glycol, unlabelled PAPS and 0.05 ml enzyme in a final volume of 0.10–0.15 ml. Controls contained 0.005 ml of propylene glycol in place of the DHEA and counts obtained were subtracted from the counts recorded in the presence of steroid. Details as used in particular experiments are noted in figure legends. In early experiments ^{35}S-labelled steroid sulphates were also estimated by chromatography (Solvent A) of 0.05 ml from the supernatant of the incubation mixture. The amount of [^{35}S]PAPS in these experiments was increased 5-fold, and the product was estimated from the area under the curve after scanning.

Protein assay

Protein was determined by measurement of absorbances at 260 and 280 mμ (ref. 24).

Gel filtration

Gel filtration on Sephadex G-200 (bead form) was carried out at 5°, initially on a small column (32 cm × 1 cm) and later on a larger column (95 cm × 1.5 cm). The columns were equilibrated with 0.05 M Tris–HCl buffer (pH 7.5) for 24 h. This buffer was also used as eluant. For the small column, enzyme supernatant (= 0.5 g tissue) was concentrated to 0.5 ml by addition of dry Sephadex G-25. For the large column, enzyme supernatant (= 3.0 g tissue) was concentrated to 1 ml by dialysis against 20% (w/v) polyethylene glycol 20 000 (Carbowax 20 M, Union Carbide) in 0.05 M Tris–HCl buffer (pH 7.5) at 5° for 5 h. Two-ml fractions were collected and these tested for DHEA sulphotransferase activity as described above and under RESULTS. Column void volumes were measured using blue Dextran 2000. Flow rates were 10 ml/h (small column) and 8 ml/h (large column).

Sucrose gradient centrifugation

The method of MARTIN AND AMES[10] was employed and the apparatus used for preparing the gradients was that of BRITTEN AND ROBERTS[11], as modified by the former authors. After preparing the gradients from 5% and 20% (w/v) sucrose solutions prepared in 0.05 M Tris–HCl buffer (pH 7.5), high speed enzyme supernatant (0.1–0.2 ml) containing 2–3 mg protein was gently layered onto the gradient. Centrifugation was conducted in the swing head of a Christ (Model Ω) preparative ultracentrifuge. Speeds of 30 000 rev./min were used which gave 97 000 $\times$ *g* at the base of the tube. The chamber temperature was set at -5° and the head temperature rose to about 10° over a 20-h run. A trial run with standard proteins suggested that centrifugation for 20 h was required to give optimal separation of proteins with molecular weights from 50 000 to 400 000. This time was used in all subsequent runs. At the completion of a run the tube was placed in a supporting device and punctured by forcing it onto a clamped twin-pointed needle connected to 1-mm internal diameter plastic tubing. Ten-drop fractions were collected and examined for DHEA sulphotransferase activity as described under RESULTS. Gradient formation and fraction collection were carried out in the cold room.

RESULTS

Estimation of product formation by the WENGLE *and chromatographic procedures*

Preliminary experiments were aimed at some degree of purification of the enzyme. Using $(NH_4)_2SO_4$ fractionation no useful degree of purification was achieved and the fractionated enzyme rapidly lost activity when kept at 0°. Due to the restricted amount of tissue available and to the uncertainty of supply, studies have been carried out in the main with high speed supernatants, without further purification. Sulphotransferase preparations, even when partially purified, can contain bound substrate which will form labelled ester sulphates when incubated with [^{35}S]PAPS of high specific activity[6]. This can be readily demonstrated by paper chromatographic techniques[6]. The WENGLE method of assay of sulphotransferases measures total [^{35}S]ester sulphates remaining in solution after $^{35}SO_4^{2-}$ and [^{35}S]PAPS have been precipitated as insoluble barium salts[9]. Using relatively high substrate concentrations and low concentrations of enzyme, the problem of the formation of sulphate esters of endogenous acceptors is not serious. However when low concentrations of [^{35}S]PAPS (0.05 mM) were used in the presence of propylene glycol in place of DHEA, appreciably high "blank" values were obtained by the WENGLE procedure*. That this radioactivity represented [^{35}S]ester sulphates, probably formed from endogenous acceptors, was shown by (i) addition of Zn^{2+}, which completely inhibited DHEAS formation (see below) reduced the radioactivity (assayed by the WENGLE method) to the very low levels obtained by following the assay procedure in the absence of the enzyme, and (ii) examination by paper chromatography and scanning showed the presence of minor peaks. Addition of DHEA resulted in the formation of [^{35}S]DHEAS which was identified on chromatograms by reference to authentic material. The WENGLE method of assaying the steroid sulphate, formed at low concentrations of DHEA, is compared to the formation of DHEAS, as shown by paper chromatography, in Fig. 1.

* High blanks were not due to sulphation of propylene glycol since replacement of the latter with water yielded approximately the same number of counts.

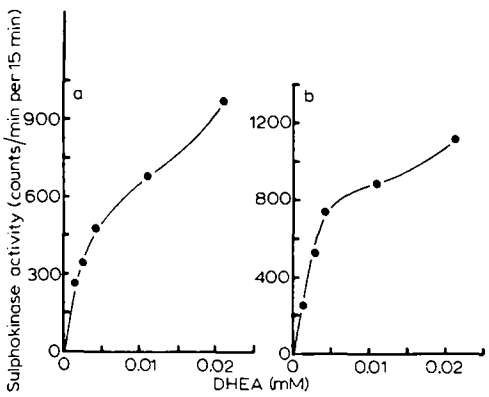


Fig. 1. Comparison of [^{35}S]DHEAS formation at low concentrations of DHEA by means of chromatography (a) and the WENGLE method (b). PAPS, 0.10 mM; incubation time, 15 min. Aliquots (0.05 ml) were chromatographed on paper in Solvent A and the DHEAS zones cut out and counted by liquid scintillation. The remaining incubation samples were then analysed by the WENGLE procedure. Results obtained by the latter method are expressed as radioactivity in 1 ml of supernatant after addition of $Ba(OH)_2$ and H_2SO_4 and have been corrected by subtraction of counts obtained with a control employing propylene glycol in place of steroid (see METHODS).

Formation of labelled products in the absence of added steroid was most significant when [^{35}S]PAPS of very high specific activity, *i.e.* prepared from carrier-free $^{35}SO_4^{2-}$, was used which again suggests that such compounds arise from endogenous acceptors present in limiting concentration. In general their formation was a major source of interference only at low concentrations of substrates (DHEA < 0.02 mM; PAPS < 0.05 mM). The WENGLE method was used in initial kinetic experiments and due to the very pronounced alterations which occurred in initial velocity-substrate curves under the influence of various additives, it was felt justified in retaining this method rather than employ the tedious and time-consuming alternative method of paper chromatography. The WENGLE method itself was shown to give a reproducibility of about $\pm$ 10% by assay of 10 individual, but identical, incubation mixtures.

pH optimum

Using both DHEA and pregnenolone as substrates, the pH optimum employing Tris–HCl buffers, was 7.7 (Fig. 2). This differs slightly from the value reported previously[7] using phosphate buffers (pH 7.4), but is much higher than the value of about 6 reported by BOSTRÖM AND WENGLE[12] using citrate–phosphate buffer. BANERJEE AND ROY[13] have reported the pH optimum as 7.5 for the sulphation of DHEA by guinea-pig liver preparations.

Effect of time of incubation

Fig. 3 shows the effect of time of incubation on product formation at different concentrations of steroid and PAPS.

Sulphation of other steroids

While the number and specificity of individual sulphotransferases in the human adrenal must await purification studies, BANERJEE AND ROY[13], using guinea-pig liver,

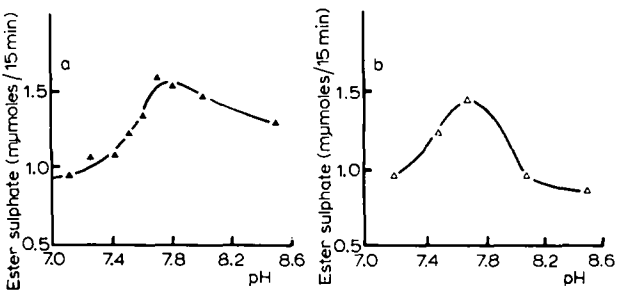


Fig. 2. pH optima using DHEA (a) and pregnenolone (b) as substrates. Concentrations of substrates were: PAPS, 0.05 mM; steroid, 0.056 mM. Incubation time, 15 min. Tris HCl buffers (0.1 M) employed throughout.

have indicated that the number of individual enzymes may be limited since constant relative activity upon progressive purification was obtained with DHEA, cholesterol, androsterone and testosterone as substrates. Some results pertaining to the sulphation of 3β-hydroxy-Δ^5-steroids by human adrenal extracts are shown in Table I.

In Expts. 1 and 2 of Table I, paper chromatography in Solvent A of tubes con-

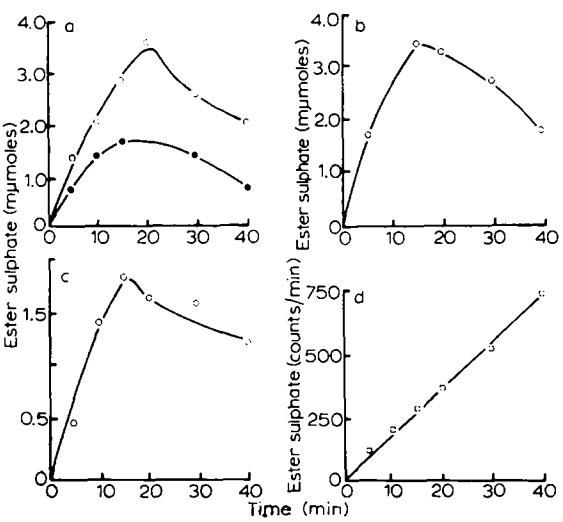


Fig. 3. Effect of time of incubation. a. PAPS, 0.15 mM; DHEA, 0.01 mM (●), 0.07 mM (○). b. PAPS, 0.10 mM; DHEA, 0.07 mM. c. PAPS, 0.05 mM; DHEA, 0.07 mM. d. PAPS, 0.20 mM; DHEA, 0.10 mM using an $(NH_4)_2SO_4$ fraction (0.35–0.55 satn.). In Expts. a–c, a high speed supernatant enzyme was used.

taining pregnenolone showed the appearance of a new zone with an R_F slightly greater than DHEAS. In the case of cholesterol, formation of cholesteryl sulphate was not significant in Expts. 1 and 2, but was significant in Expt. 3 where EDTA was substituted for Mg^{2+}. The WENGLE method of assay was used in these experiments. Paper chromatography of aliquots from tubes of Expt. 3 showed the formation, in the presence of cholesterol, of a new zone with a high R_F which corresponded to authentic

TABLE I

RELATIVE SULPHATION OF 3β-HYDROXY-Δ^5-STEROIDS

Expt. No.	*PAPS (mM)*	*Steroid*	*Steroid Concn. (mM)*	*Sulphation** (%)
1	0.16	DHEA	0.008 (0.08)	100 (100)
	Mg^{2+} (10 mM)	Pregnenolone	0.008 (0.08)	46 (68)
		Cholesterol	0.008 (0.08)	1 (4)
2	0.05	DHEA	0.008 (0.08)	100 (100)
	Mg^{2+} (10 mM)	Pregnenolone	0.008 (0.08)	33 (27)
		Cholesterol	0.008 (0.08)	1 (1)
3	0.05	DHEA	0.008	100
	No Mg^{2+} EDTA (10 mM)	Cholesterol	0.008	13

* Relative to sulphation of DHEA = 100%.

cholesteryl sulphate. Conditions in Expt. 3 were similar to those used by BANERJEE AND ROY[13], and while the definite formation of cholesteryl sulphate needs further confirmation, it may be significant that the relative rate of sulphation compared to DHEA (13%) was similar to that reported by these workers[13].

Kinetic studies

The WENGLE method of assay was used for all kinetic studies and as outlined above would be subject to error due to sulphation of endogenous acceptors, especially at low substrate concentrations. In addition, when employing high speed supernatant enzyme, the choice of 15-min incubation periods, as used in the majority of studies, does not give a strict measure of the initial velocity as can be seen from Fig. 3. However the gross departure from Michaelis–Menten kinetics (Figs. 4–7) certainly cannot be explained by these influences alone. That the kinetic results obtained with the enzyme supernatant, represent the properties of a "real" system, giving rise to altered rates of formation of DHEAS in response to changes in DHEA and PAPS concentrations, is supported by the following facts: (i) a single peak enzyme fraction obtained by gel filtration on Sephadex G-200 gave very similar kinetic results to that obtained with the crude supernatant (*c.f.* Fig. 4); (ii) different high speed supernatant preparations gave a fair degree of reproducibility in the kinetic curves, with peaks occurring at about the same concentrations of DHEA; (iii) purified estrogen sulphotransferase, Form A, which gave normal Michaelis–Menten kinetics, yielded complex curves when association was induced by addition of cysteine to the system[5]; (iv) physico-chemical studies (described below) revealed a system which could be expected to exhibit quite complex kinetic behaviour. Kinetic data will be presented in light of the above remarks.

Fig. 4a shows the effect of varying the DHEA at constant low levels of PAPS (0.05 mM), using crude supernatant enzyme. In Fig. 4b, the enzyme was obtained from tissue stored frozen for 12 months. Similar curves, obtained with a single peak fraction and from pooled A fraction from Sephadex G-200 filtration (*c.f.* Fig. 9), is shown in Fig. 4c.

The most simple general explanation of the kinetics shown in Fig. 4 would be

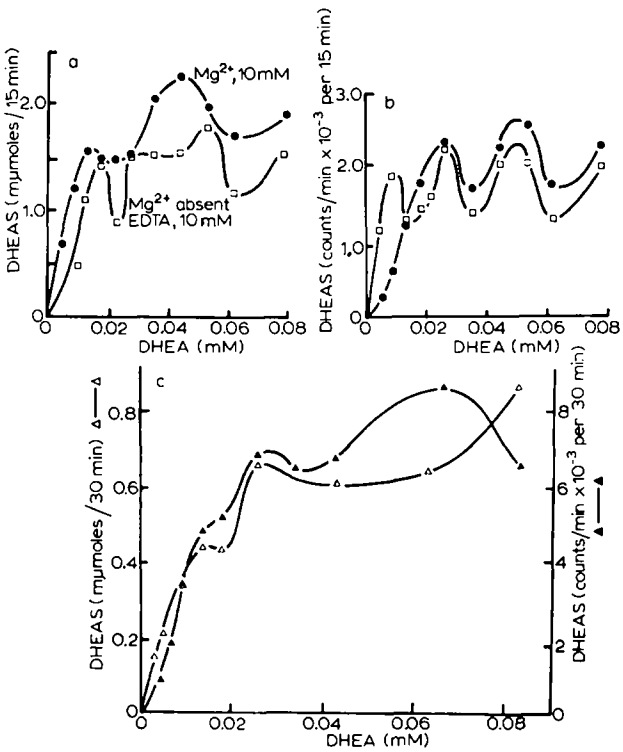


Fig. 4. Kinetics with DHEA as variable substrate. a. High speed supernatant enzyme, PAPS 0.05 mM. Incubation time, 15 min. b. High speed supernatant from tissue stored frozen for 1 year, PAPS 0.05 mM. Incubation time, 15 min. ●, DHEA alone; □, DHEA *plus* cysteine, 20 mM. c. ▲, peak fraction A enzyme from Sephadex gel filtration (see Fig. 9b) after standing 48 h at 0°. PAPS, 0.07 mM. Incubation time, 30 min. △, pooled Fraction A after standing 2 weeks at 0°. PAPS, 0.05 mM. Incubation time, 30 min.

that the curves represent not a single enzyme species, but the compounded activities of a series of enzyme forms. Complex kinetics obtained with crude hexokinase from human liver for example, have been explained in terms of the co-existence of two distinct species of enzyme with markedly different kinetic properties[15]. In the Lineweaver–Burk plot (Fig. 5), obtained from data in Fig. 4c, portions of the curve can be extrapolated to give a series of increasing apparent K_m values (0.016, 0.04, 0.10 mM), which could be interpreted as corresponding to different enzyme species.

The concentration of PAPS was shown to have a marked effect on the kinetics obtained with varying DHEA—both with crude supernatant and with fractions obtained by gel filtration. High concentrations of PAPS changed both the relative size and the position of the peaks. This can be seen in Fig. 7, and on comparison of Fig. 6 with Fig. 4. Addition of ascorbate to incubations at these high PAPS levels is also shown in Fig. 6. This is seen to produce a curve similar to that normally obtained at low PAPS concentrations and its effect on the system would seem to be in reversing the nature or proportion of enzyme forms favoured by high PAPS concentrations.

Kinetic behaviour exhibited by the Fractions A and B, obtained by gel filtration (*c.f.* Fig. 9), and stored at 0° for 2 weeks, showed that neither fraction behaved as a

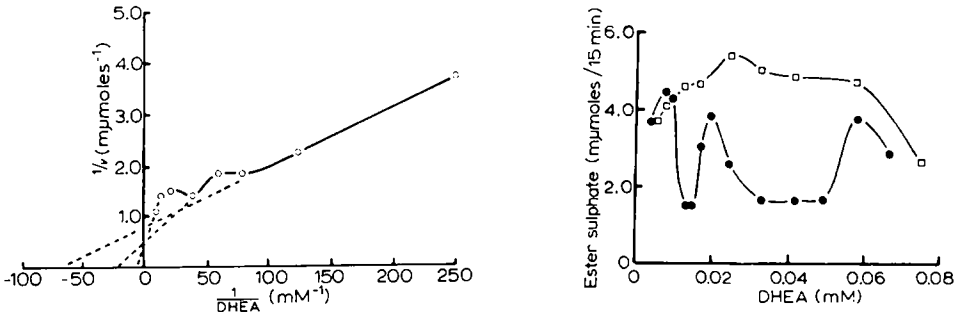


Fig. 5. Lineweaver Burk plot obtained from data shown in Fig. 4c, employing pooled Fraction A from Sephadex gel filtration.

Fig. 6. Kinetics with DHEA as variable substrate at higher concentrations of PAPS in the presence and absence of ascorbate. High speed supernatant enzyme. PAPS, 0.14 mM. Incubation time, 20 min. ●, DHEA alone; □, DHEA plus ascorbate (10 mM). In this experiment total ester sulphate is recorded; values have not been corrected by subtraction of controls carried out in the absence of added steroid.

single enzyme species. Indeed the main fraction (A), as shown in Fig. 7a, produced kinetic data similar to that obtained with the original high speed supernatant (*c.f.* Fig. 4a). The effect of varying PAPS at constant but differing concentrations of DHEA is shown for Fraction A in Fig. 7b.

Metal ions

The behaviour of various metal ions on the activity of the enzyme was generally very similar to that described previously with estrogen sulphotransferase[4]. At concentrations of 10 mM (PAPS, 0.05 mM; DHEA, 0.01 mM; incubation 15 min) both Mg^{2+} and Mn^{2+} activated the system to a slight extent (from 10 to 20%), although the effects were variable from experiment to experiment. Zn^{2+} was strongly inhibitory, Ni^{2+}

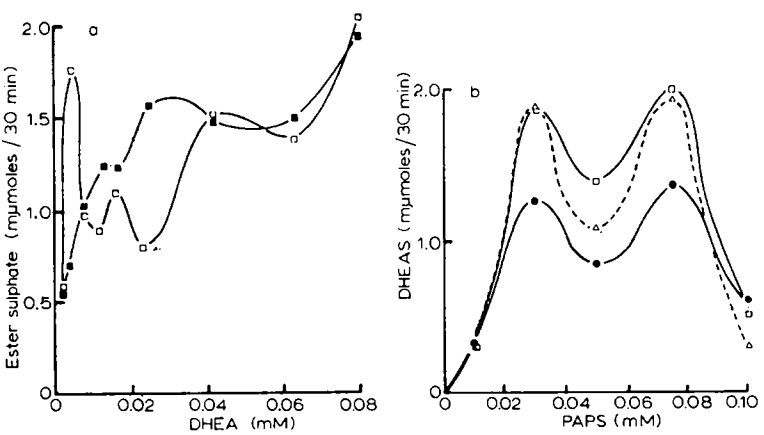
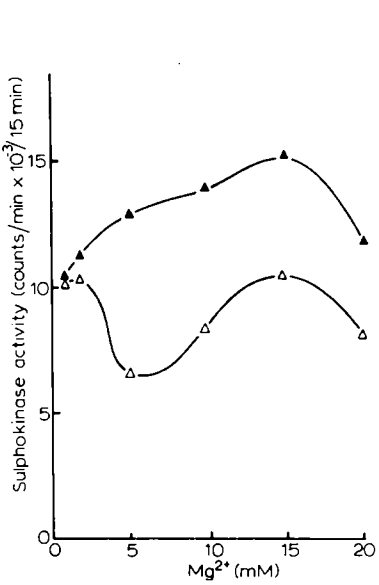


Fig. 7. Kinetics with Fraction A enzyme obtained by gel filtration. This fraction had stood for 2 weeks at 0°. a. DHEA as variable substrate. PAPS, 0.05 mM (■), 0.20 mM (□). In this case total ester sulphate formation is recorded; values have not been corrected (see Fig. 6). b. PAPS as variable substrate. DHEA, 0.008 mM (●), 0.017 mM (△), 0.063 mM (□). Normal assay conditions employed in b with the exception that in both a and b incubation time was extended to 30 min.



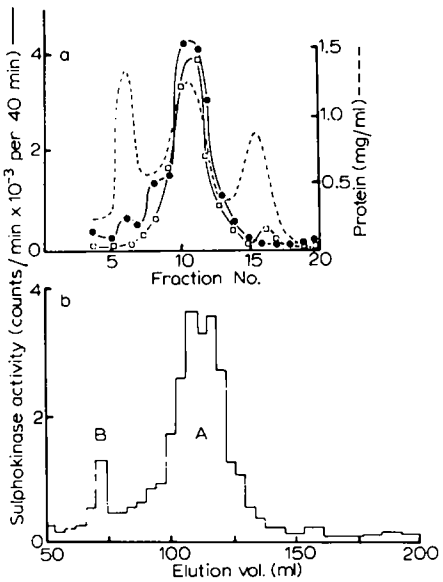


Fig. 8. Effect of Mg^{2+} on enzyme activity. High speed supernatant enzyme. PAPS, 0.05 mM. DHEA, 0.01 mM (△), 0.06 mM (▲). Incubation time, 15 min.

Fig. 9. Gel filtration on columns of Sephadex G-200. a. Small column (see METHODS). Assay conditions were: 0.1-ml column fraction; PAPS, 0.11 mM; Mg^{2+}, 12 mM; DHEA, 0.01 mM (●) and 0.11 mM (□). Total vol., 0.125 ml. Incubation time, 40 min. Protein was monitored automatically by absorption at 255 mμ and the concentration in each fraction determined by absorbance measurements at 280 and 260 mμ (ref. 24). b. Large column (see METHODS). Assay conditions were: 0.1-ml column fraction; PAPS, 0.10 mM; Mg^{2+}, 10 mM; DHEA, 0.01 mM. Total vol., 0.135 ml. Incubation time, 40 min.

inhibited by 20% and Co^{2+} had no effect at the substrate concentrations quoted above. At concentrations of 30 mM, Na^+ had no effect, whereas K^+ exhibited some activation (20%). Effects of increasing concentrations of Mg^{2+}, at two levels of DHEA, are shown in Fig. 8. The unusual shape of the "activation" curves suggest an indirect effect on activity rather than direct participation at the active site. Some support for the concept that sulphation at different substrate levels may be predominately due to distinct enzyme species is provided by the differential effects of Mg^{2+} at the two DHEA levels.

Possible effects on sulphydryl groups of the enzyme

The effects of cysteine and ascorbate on the kinetics under defined conditions have already been described (Figs. 4 and 6). These reagents, together with *p*-chloromercuribenzoate (PCMB), were then examined in the one experiment at low PAPS concentration and two alternative concentrations of DHEA (Table II). Cysteine and ascorbate exhibited similar effects; activating more markedly at low steroid concentration. PCMB had the reverse effect in showing stronger inhibition at the low steroid level. Because of the similar behaviour of cysteine and ascorbate, a specific chemical (*i.e.* allosteric) combination of ascorbate* with enzyme seems unlikely. One obvious,

* Ascorbic acid occurs in bovine adrenal glands at a concentration of 1.2 mg/g (ref. 14). Concentrations used in the above experiments would be of this order.

but possibly erroneous interpretation, is that a free SH group(s), maintained by the reducing environment and blocked by PCMB, is required for maximum activity. Again these results show close similarity with those described for estrogen sulphotransferase[4,5].

Other effects on activity

In considering possible roles of steroid alcohol sulphotransferase as a control enzyme, one mechanism could be by way of feed back control by the end products of biosynthetic pathways. The effects of low concentrations (0.01 and 0.02 mM) of a number of steroid hormones on enzyme activity (PAPS, 0.05 mM; DHEA, 0.06 mM)

TABLE II

EFFECTS OF REDUCING AGENTS AND –SH BLOCKING AGENTS

PAPS 0.05 mM. Change in activity was determined from the total radioactive ester sulphates produced, *i.e.* DHEAS and ester sulphates formed from endogenous acceptors.

Addition	*Concn. (mM)*	*% Change in activity*	
		0.01 mM DHEA	*0.06 mM DHEA*
Cysteine	5	+18	+19
	10	+24	+18
	20	+30	−20
Ascorbate	5	+16	+12
	10	+37	+14
	20	- -	+ 7
PCMB	1	−10	± 0
	10	−83	−58

were examined. Of the steroids tested (corticosterone, cortisol, cortisone, progesterone, estrone, 17β-estradiol, estriol and estrone sulphate), only estrone and progesterone had significant effects. They were slightly inhibitory (< 20%). The adenine nucleotides AMP, ADP and ATP were also examined at low concentrations (0.01 and 0.02 mM) with substrate concentrations: PAPS, 0.05 mM; DHEA, 0.01 and 0.06 mM. At 0.01 mM DHEA, AMP inhibited slightly and ATP activated slightly (<20%), but all other effects observed were <10%.

Physico-chemical studies

Gel filtration

Because of (a) the existence of at least two forms of estrogen sulphotransferase separable by column chromatography[5] and (b) the likelihood that the kinetic results could possibly be explained by the presence of discrete enzyme species exhibiting different activities, effort was then made to demonstrate the presence of more than one enzyme form. Fig. 9 shows the results obtained using high speed supernatant enzyme and Sephadex G-200 columns. If separate molecular weight species did in fact possess differing kinetic properties, it was considered that improved resolution may have been possible by assaying effluent fractions at two substrate levels, *e.g.*

TABLE III

RELATIVE ACTIVITIES OF PEAK FRACTIONS FROM SMALL SEPHADEX G-200 COLUMN

Fractions 6, 8 and 10 from the gel filtration shown in Fig. 9a were compared at four concentrations of DHEA. The volume of enzyme used was 0.05 ml in a final volume of 0.09 ml. PAPS, 0.3 mM. Assay after fractions had stood 36 h at 0°.

Fraction No.	*Relative activities of fractions (counts/min per 20-min incubation)*			
	0.01 mM DHEA	*0.015 mM DHEA*	*0.02 mM DHEA*	*0.05 mM DHEA*
6	483	3860	318	334
8	1210	1330	3890	1060
10	2790	2780	3370	2910

high and low concentrations of DHEA. As can be seen, the assay at high DHEA gave a symmetrical peak, whereas assays at 0.01 mM DHEA gave, as well, two smaller peaks corresponding to higher molecular weight species.

The relative activities of three peak fractions from the smaller column (Fig. 9a) were compared at four DHEA concentrations. Results are shown in Table III.

The larger column (Fig. 9b) gave two well-defined peaks and rough calibration of this column with protein standards showed that these peaks corresponded to molecular weights of about 70 000 (Peak A) and 130 000 (Peak B).

Sucrose gradient centrifugation

Sucrose gradient centrifugation in the preparative ultracentrifuge provided an alternative means of examining for enzyme forms of different molecular weight and for establishing any possible relationship between these forms. Experiments using this technique were confined to the use of high speed supernatant enzyme. Results of a 20-h centrifugation in 0.05 M Tris–HCl buffer (pH 7.5) are shown in Fig. 10. DHEA sulphotransferase activity was distributed through many fractions in contrast to haemoglobin (also present in the crude enzyme) which was confined to a fairly narrow region of the tube. The enzyme sedimentation behaviour, then, suggested a mixture of different molecular weight species. Although separation was incomplete, it was possible to identify three major regions of activity (A', B' and C'). Utilising haemoglobin (mol.wt. 64 500) as a reference, molecular weights corresponding to each of these regions were calculated using the expression

$$\frac{X_1}{X_2} = \left(\frac{\text{mol. wt.}_1}{\text{mol. wt.}_2}\right)^{2/3} \quad (1)$$

where X_1/X_2 is the ratio of distances moved from the meniscus by Proteins 1 and 2 (ref. 10). NOLL[16] has warned that application of Eqn. 1 other than in the exact conditions used by MARTIN AND AMES[10], may lead to significant errors in calculated molecular weights. Conditions used in this study were almost identical except that the speed used (30 000 rev./min) was less than the 38 000 of MARTIN AND AMES[10]. It was therefore desirable to check the validity of Eqn. 1 by centrifuging a second protein of known molecular weight. Catalase was chosen, and its distribution under identical conditions as used with the sulphotransferase, is shown in Fig. 10. Excellent

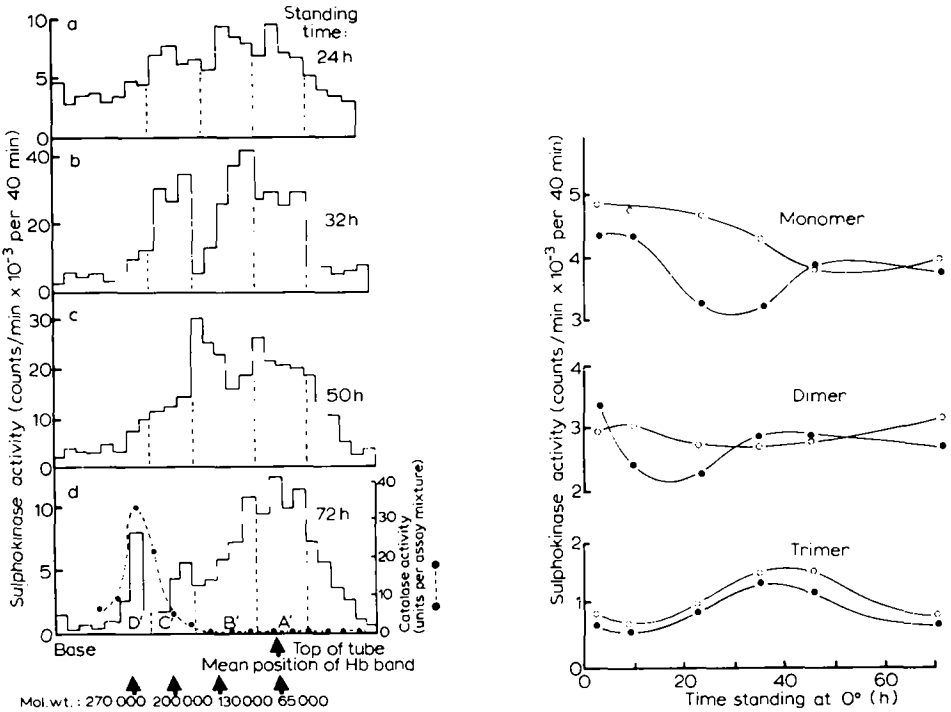


Fig. 10. Sucrose gradient centrifugation of high speed supernatant enzyme. Conditions of centrifugation and fraction collection are described in METHODS. The times of standing at 0° between fraction collection and assay are shown. Assay conditions: in a and d, 0.05 ml of each fraction was incubated for 40 min with PAPS (0.05 mM) and DHEA (0.01 mM) in a final volume of 0.15 ml. In b and c, 0.2 ml of each fraction was incubated for 40 min with PAPS (0.06 mM) and DHEA (0.01 mM) in a final volume of 0.23 ml. Because of variation in amount of enzyme and specific activities of ^{35}S-PAPS solutions, the relative ordinate scales have no significance. The distribution of catalase, run as a standard protein under identical conditions, is also indicated. Catalase activity was measured spectrophotometrically[25]. Hb, haemoglobin (present in the adrenal enzyme).

Fig. 11. Changes in activity of transported species during return to equilibrium. Samples (0.2 ml) of high speed supernatant enzyme were subjected to sucrose density-gradient centrifugation in 3 separate tubes. Aliquots from fractions corresponding to monomer (A'), dimer (B') and trimer (C') (see Fig. 10) were pooled and allowed to stand at 0°. Enzyme activity was then determined on these pooled fractions after various time intervals. Assay conditions: PAPS, 0.05 mM; DHEA, 0.01 mM (●), 0.05 mM (○). Enzyme 0.05 ml, in a final volume of 0.15 ml. Incubation time, 40 min.

agreement between published data[17] for the molecular weight of catalase (248 000) and that calculated by reference to haemoglobin was obtained (Fig. 10). The calculated molecular weights for the main DHEA sulphotransferase species were then: A', 65 000; B', 130 000; C', 200 000. A fourth peak D', corresponded to a molecular weight of about 270 000. Thus ultracentrifugation confirmed and extended the results of gel filtration by providing evidence for a number of enzyme species of differing molecular weight. Values for the latter were consistent with the idea that these species represented aggregates of a similar monomeric unit, related as monomer, dimer, trimer, *etc.* The

existence of estrogen sulphotransferase in two or more different states of association[5] provided a precedent for this finding.

The "qualitative" distribution of enzyme activity in the sedimentation patterns in Fig. 10 was similar in all cases but the quantitative agreement was rather poor, *i.e.* the relative activities in A', B', C' (and D') regions differed from experiment to experiment. Consideration of the procedures used suggested that these differences in activity might be explained by the different times of standing employed in each experiment, *i.e.* the different intervals between collection of fractions after centrifugation and the time these were assayed for enzyme activity. In turn, such a dependence of activity in the ultracentrifuge fractions upon their time of standing, could be explained if various enzyme forms with different activities existed in a slowly reversible association–dissociation equilibrium. As a result of the partial separation of

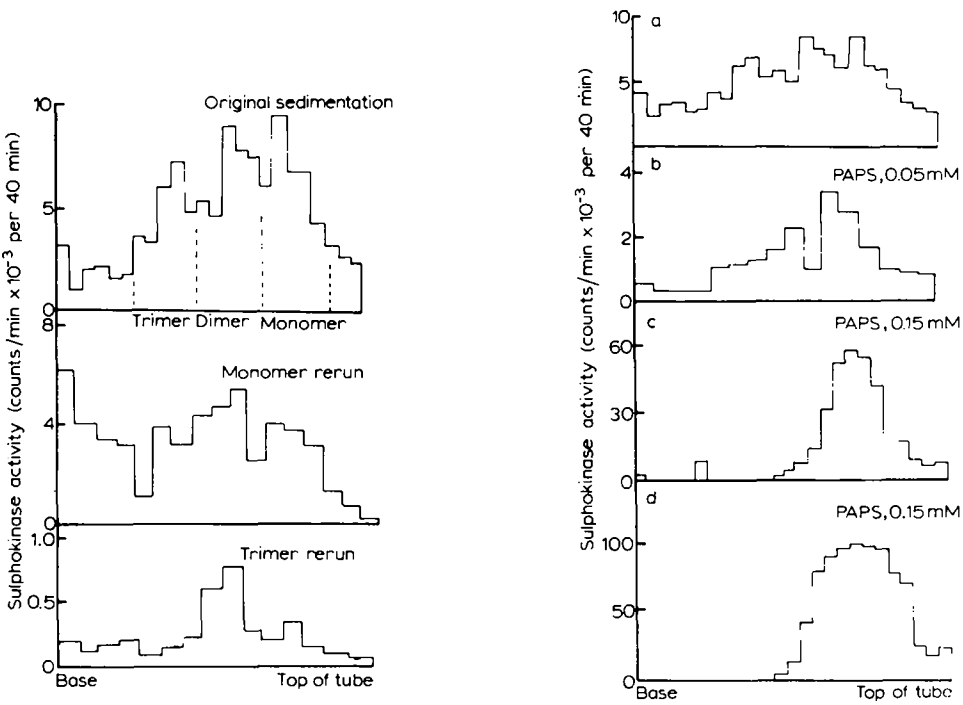


Fig. 12. Evidence for the existence of a reversible association–dissociation equilibrium. Data were obtained from the centrifugal run described in Fig. 11. The complete sedimentation pattern was determined after 24 h and is shown at the top of Fig. 12. The pooled monomer, dimer and trimer fractions (see Fig. 11) were allowed to stand for 72 h and then dialysed against repeated changes of 0.05 M Tris–HCl buffer (pH 7.5) for 4 h. Concentration was then achieved by dialysis against 20% (w/v) polyethylene glycol for 6 h. Monomer and trimer fractions were then recentrifuged and the resulting sedimentation patterns were determined after standing for 24 h. Assay conditions as in Fig. 10a.

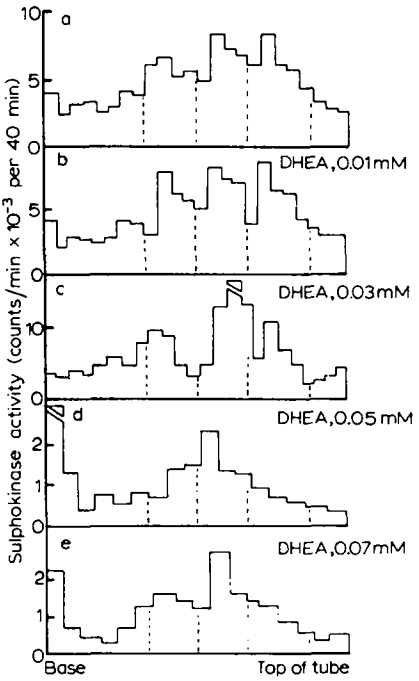
Fig. 13. Effect of PAPS on sedimentation pattern. In b–d, PAPS was added to enzyme sample and sucrose solutions to give the concentrations shown. In d, the enzyme was incubated for 15 min at 37° in the presence of 0.15 mM PAPS, prior to sedimentation. Assay conditions as in Fig. 10a which is also included for comparison (a). Relative ordinate scales have no significance (see Fig. 10).

species in the centrifuge, the proportion of monomer, dimer, *etc.* in any fraction immediately after sedimentation would not be identical to the normal equilibrium distribution. If the various forms were interconvertible the enzyme would revert to the normal equilibrium on standing and changes in activity with time could thus be related to changes in the proportions of monomer, dimer, *etc.* Results of an experiment designed to test this hypothesis are shown in Figs. 11 and 12. From an initial run, fractions corresponding to monomer, dimer and trimer were assayed at two different steroid levels after various times of standing at 0°. After 72 h, the monomer and trimer fractions were subjected to a second run. It was found (Fig. 11) that the activities of the three fractions did vary with time of standing in a way which correlated reasonably well with the changes in sedimentation pattern observed in Fig. 10. The recentrifugation of monomer and trimer showed (Fig. 12) that, in both cases, re-equilibration had occurred to give a distribution of enzyme similar to the original sedimentation pattern, which was assayed after 24 h standing. Evidence was then provided for the existence of a slowly reversible association–dissociation equilibrium and for a relationship between state of association and activity.

From the complex kinetics exhibited by DHEA sulphotransferase, it was possible that either, or both, substrates could modify the activity of the enzyme. Such effects may have been paralleled by changes in the position of the association–dissociation equilibrium. The sedimentation patterns formed in the presence of various concentrations of one or other substrate are shown in Figs. 13 and 14. High concentrations of PAPS (0.15 mM) caused marked dissociation—nearly all of the enzyme existing as monomer (Fig. 13c). When the enzyme was incubated at 37° for 15 min in 0.15 mM PAPS prior to centrifugation, the sedimentation pattern was essentially the same (Fig. 13d). The equilibrium position was displaced only slightly towards dissociation by 0.05 mM PAPS (Fig. 13b). Unlike PAPS, DHEA favoured association. Although its effects were less dramatic, there was a definite trend in sedimentation patterns towards association with increasing concentrations of steroid (Fig. 14). At 0.05 and 0.07 mM DHEA, there was a marked increase in the relative activity in fractions corresponding to molecular weights $>300\,000$, suggesting the formation of higher states of association.

Mg^{2+} was observed to favour association as is shown in Fig. 15. This provides a basis for explaining the effects of Mg^{2+} in Fig. 8. The fall, followed by a rise in activity at low steroid concentration, for instance, could be due to progressive changes in the degree of association of the enzyme—initially to a less active, then further to a more active species. It is important to note that the assay conditions used for estimating sedimentation patterns were: 0.05 mM PAPS, 0.01 mM DHEA and 10 mM Mg^{2+}. At these levels only Mg^{2+} has a very significant effect on sedimentation pattern. The associative effect of Mg^{2+} would be offset to some degree by the dissociative effect of PAPS. Since changes in activity could be detected after the separated species were allowed to stand (Fig. 11), this itself indicates that the assay conditions did not impose a fixed equilibrium condition and that the pre-existing state of association was altered only slightly during the assay procedure.

The effect of cysteine on sedimentation behaviour was also examined since it activated the enzyme (Table II) and was known to favour association with estrogen sulphotransferase[5]. As shown in Fig. 15, 30 mM cysteine caused considerable association of the enzyme, and states of association higher than tetramer were evident.



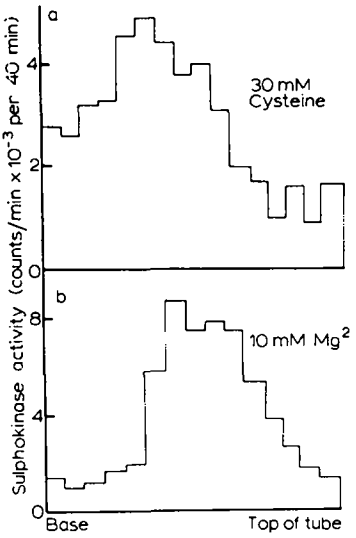


Fig. 14. Effect of DHEA on sedimentation pattern. In b–e, DHEA (in a constant volume of propylene glycol) was added to enzyme sample and sucrose solutions to give the concentrations shown. Assay conditions: b and c as in Fig. 10a; d, as in Fig. 10a but the fractions were diluted to give the desired final DHEA concentration; e, the assay was performed after the separated fractions had stood for 48 h at 0° and contained 0.025 ml of centrifuge fraction, 0.05 mM PAPS and 0.012 mM DHEA in a final volume of 0.15 ml (incubation time, 40 min). Fig. 10a is included for comparison. Relative ordinate scales have no significance (see Fig. 10).

Fig. 15. Effect of cysteine and of Mg^{2+} on sedimentation pattern. These agents were added to the enzyme sample and sucrose solutions to give the concentrations shown. Assay conditions: a as in Fig. 10a. b, as in Fig. 10b, except that assays were performed after the separated fractions had stood for 30 h at 0°. The relative ordinate scales have no significance (see Fig. 10).

DISCUSSION

Despite the limitations imposed on the results obtained in the kinetic experiments, which would preclude any attempt at interpretation by rigorous kinetic theory, the gross departure from Michaelis–Menten behaviour must be considered as reflecting real properties of the enzyme system. It was previously suggested that the kinetic results might be explained by the presence of a mixture of enzyme forms of varying activities, and furthermore that the proportion of these forms might be dependent on substrate concentrations and other "environmental" factors. This interpretation received good support from the physicochemical studies whereby the presence of such forms, which existed in a reversible association–dissociation equilibrium, was established. The very pronounced differences which occur in the kinetics at low (0.05 mM) and high (0.15 mM) PAPS concentrations can be correlated with the change in the position of the normal equilibrium to favour the dissociated state (Fig. 13). The undulating nature of the kinetic curves may be due, in part, to altered proportions of

the various forms of the enzyme occurring during the 15-min incubation period. Such proportions would be governed by the differing ratios of PAPS and DHEA—the latter, at increasing concentration tending to shift the equilibrium towards the associated state. A relationship between activity and state of association would then be indicated.

The fact that Fraction A, obtained by gel filtration and examined after 1–2 weeks standing at 0°, did not behave kinetically as a single enzyme species (Fig. 7) could be explained by the restoration of the normal equilibrium. When monomer and trimer fractions, obtained by transport in the centrifugal field, were allowed to stand and recentrifuged then the new sedimentation patterns were somewhat different; the monomer fraction had re-equilibrated to yield a greater proportion of associated species than had the trimer fraction (Fig. 12). These differences, after equilibration, may indicate that the association–dissociation equilibrium is dependent on protein concentration since the transported monomer fraction contained about 5 times as much enzyme protein as the trimer (see below). Re-formation of the A form which occurred when the isolated B form of estrogen sulphotransferase was rechromatographed on DEAE-cellulose[5], would also be explained on the basis of an equilibrium governing an association–dissociation reaction in this enzyme system.

More direct evidence for a relationship between activity and degree of association is the fact that components other than substrate can affect association and also cause significant changes in enzyme activity. Both cysteine and Mg^{2+} cause association (Fig. 15), and both activated the enzyme (Table II, Fig. 8). It is perhaps also significant that these and other modifiers of enzyme activity exert differential effects on activity, *i.e.* they activate or inhibit to a significantly different extent at different substrate levels—supporting the concept that distinct enzyme species are responsible for sulphation at various substrate levels. One other explanation of the results could be due to the existence of a number of individual steroid sulphokinases with overlapping specificities and thus varying activities as regards the substrate DHEA. Perhaps the most important piece of evidence that this is not the case—and indeed lends strong support to the ideas presented above for a relationship between activity and state of association within a single enzyme system, is presented in Fig. 11. Independent of any modifying agent, which could cause conformational changes and possible changes in activity—with association following as a secondary and non-obligatory consequence, an alteration in the activity of the separated species was observed on standing. The only known variable to account for such alteration in activity is the change in proportion of the molecular species as the association–dissociation equilibrium, disturbed by transport of the species in the centrifugal field, is restored. Some information concerning the relative proportions of monomer, dimer and trimer existing in the original enzyme supernatant can also be gained from this experiment (Fig. 11). Assuming that all three fractions reach the same equilibrium* after 70 h standing, then the relative amounts of enzyme protein in each fraction is proportional to the final activity. This leads to the following ratios: monomer:dimer:trimer = 5:4:1.

The interconversion of the enzyme species at 0° and in the absence of substrates *etc.* is evidently very slow, requiring some 70 h. Although such equilibria in polymerising systems have been dealt with theoretically[18], it is believed that this is the first example of an enzyme system governed by such a low rate constant. This raises the question as

* This is only approximately true as can be seen from the data in Fig. 12 concerning the recentrifuging of the monomer and trimer fractions.

to whether the changes in association caused by such agents as PAPS and cysteine are essentially instantaneous. To achieve the sedimentation pattern in Fig. 13c, the PAPS, added to the enzyme immediately before centrifuging, must cause complete dissociation in less than about 5 h of the 20-h run. However the marked alterations in activity at high PAPS concentrations in kinetic experiments (Fig. 6) might be construed as indicating "forced" changes in association during the incubation time of 15 min. Substrate inhibition effects, or conformational changes, are other possible explanations, but since cysteine and PAPS which cause association and dissociation, respectively, are also opposed in their effects on the kinetics, this would support the former explanation.

A study of the influences of cysteine, ascorbate and PCMB on activity (Table II) could suggest that a free SH group(s) is (are) necessary for maximum activity, especially at low steroid concentrations. The presence of cysteine evidently favours association, both with this enzyme system and with estrogen sulphotransferase[5]. Since monomer was observed to re-associate without addition of cysteine or ascorbate (Fig. 12), the monomer does not evidently represent a species which has undergone complete oxidation of SH groups which might prevent re-association. Rather, it would seem that the presence of a reducing environment shifts the equilibrium towards the associated state.

Substrates and modifiers, in terms of allosteric theory, combine with one or other of two possible conformations of a protein thus altering the normal equilibrium between them. Such conformations could also differ in their ability to undergo association. Thus changes in association might be considered to arise as secondary effects and by themselves would not govern enzyme activity. Evidence is presented here, however, which indicates an obligatory link between state of association and activity. The combination of substrates, or modifiers, at allosteric sites in this case could influence the direction of the final association dissociation equilibrium. Protein–protein interactions could also modify catalytic activity due to conformational changes at, or near, the active site. In this regard NICOL, JACKSON AND WINZOR[19] have provided a theoretical explanation of allosteric effects in terms of binding of small molecules to different polymeric species co-existing in equilibrium. The experiments of BENESCH, BENESCH AND MACDUFF[20], which reinstates a dissociation step in explaining the sigmoidicity of the oxygen saturation curve of haemoglobin, and the work of GERHART AND SCHACHMAN[21] on aspartate transcarbamylase, which implicates the binding of the regulatory subunit to the catalytic subunit as an allosteric effect, were quoted as examples by NICOL, JACKSON AND WINZOR[19]. A further example of protein–protein interaction is provided by insect myosin ATPase in which actin acts essentially as an allosteric activator[22]. However in a number of studies with other allosteric enzymes where there is an apparent correlation between activity and state of association, it is difficult to determine whether conformational changes in the protein chain induced by the binding of effectors, are the direct cause of altered activity, or whether the change in degree of association accompanying binding, may be responsible. The classical example is that of glutamate dehydrogenase (EC 1.4.1.3) (ref. 23). Other examples are phosphofructokinase (EC 2.7.1.11), phosphorylase a (EC 2.4.1.1) and acetyl-CoA carboxylase (EC 6.4.1.2).

The possible biological significance of the results described is difficult to evaluate since the true physiological substrate for the enzyme is not known with certainty.

However the enzyme possesses many features which would place it in the class of a "control" enzyme. The existence of associated enzyme structures whose proportion and properties are changed by substrate and environmental factors would certainly provide the basis for such control. Preliminary studies on possible feed back effects of various steroid hormones, themselves considered as end products of multienzyme biosynthetic pathways, were not very rewarding. However more exhaustive studies over wide ranges of substrate and effector concentrations will need to be carried out before definite conclusions can be drawn.

Thus while the present work provides considerable evidence that steroid alcohol sulphotransferase may be a site of cellular control, the value of such control may not become obvious until the significance of steroid sulphate formation and secretion by the adrenals is better understood.

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