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ENZYMIC SYNTHESIS OF STEROID SULPHATES

X. ISOLATION OF OESTROGEN SULPHOTRANSFERASE FROM BOVINE PLACENTA AND COMPARISON OF ITS PROPERTIES WITH ADRENAL OESTROGEN SULPHOTRANSFERASE

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SUMMARY

Oestrogen sulphotransferase (3′-phosphoadenylylsulphate:oestrone sulphotransferase, EC 2.8.2.4) was isolated from bovine placenta by the same methods developed for the purification of bovine adrenal oestrogen sulphotransferase (Adams, J. B., Ellyard, R. K. and Low, J., (1974) Biochim. Biophys. Acta 370, 160–188). The isoenzyme patterns, substrate specificity, molecular weight, amino acid composition, kinetic behaviour and effect of thiols were studied. All of these properties were identical to oestrogen sulphotransferase of adrenal origin. Evidence was provided that oestrone was bound to the purified placental enzyme.

INTRODUCTION

The purification and properties of bovine adrenal oestrogen sulphotransferase (3′-phosphoadenylylsulphate:oestrone sulphotransferase, EC 2.8.2.4) and the nature of its isoenzyme forms were described in Part IX [1] of this series (preceding paper). In their study of the enzymic sulphurylation of steroids by bovine tissues, Holcenberg and Rosen [2] reported that the bovine placenta was the most active of the bovine tissues. Its oestrogen-sulphurylating activity per unit weight of tissue was some 2–3 times greater than that of the adrenal glands. This paper describes the isolation and purification of bovine placental oestrogen sulphotransferase and compares its properties with those of the adrenal enzyme.

MATERIALS

In general, materials were as described in the preceding paper [1]. The following steroids were obtained from Sigma Chemical: dehydroepiandrosterone, 11-deoxy-

Abbreviations: *P*-Ado-*PS*, adenosine-3′-phosphate-5′-phosphosulphate; MES, 2-(*N*-morpholino)ethane sulphonic acid.

corticosterone, oestrone, 17β-oestradiol, oestriol, 17β-oestradiol-3-methyl ether, pregnenolone and etiocholanolone. Testosterone was purchased from Light and Co. *p*-Nitrophenol was obtained from British Drug Houses and recrystallised before use. Calbiochem was the supplier of 2-(*N*-morpholino)ethane sulphonic acid (MES) and glycylglycine.

METHODS

Isolation and purification

A bovine placenta was collected from the slaughterhouse and transported to the laboratory on ice. The cotelydonary structures were dissected out, weighed and either used directly or stored frozen. Extraction and purification of the placental enzyme were carried out as previously described for the adrenal enzyme [1].

High speed supernatant extracts

Bovine placental tissue and bovine adrenal cortical tissue (10 g) were homogenised in 2 vol. of 0.25 M sucrose in a Potter–Elvehjem homogeniser. The homogenate was centrifuged at 9000 $\times$ *g* for 15 min then 80 000 $\times$ *g* for 60 min in a Beckman L2-65B ultracentrifuge. The precipitating material was discarded. The high-speed supernatant contained 10–12 mg protein per ml.

Enzyme assay

The enzyme was assayed under standard conditions [1] except that the pH of the 0.1 M Tris–HCl buffer was 7.5 and not 8.0 as previously used. $MgCl_2$ was omitted from the assays.

Endogenous binding of oestrogens to purified enzyme

Enzyme (100 μg) was incubated with $6 \cdot 10^5$ cpm *P*-Ado-$P^{35}S$ in 0.1 M Tris–HCl, pH 7.5 for 2 h at 37 °C. Aliquots were subjected to paper chromatography in 0.4 M potassium phosphate, pH 6.3, for 8 h [3]. The radioactive products were detected by scanning the strips on a Nuclear Chicago Actigraph III scanner. Markers for oestrone sulphate, 17β-oestradiol sulphate and oestriol sulphate were prepared by carrying out standard enzyme incubation with 0.05 mM oestrogen, *P*-Ado-$P^{35}S$ and adrenal oestrogen sulphotransferase.

Other methods were as previously described [1].

RESULTS

Oestrogen sulphotransferase activity of tissue extracts

The oestrogen-sulphurylating activities of placenta and adrenal tissue, employing oestrone as substrate, are compared in Table I. Activity was measured in the 80 000 $\times$ *g* supernatant fractions prepared from two different placental homogenates and also from a pooled adrenal cortical homogenate. The measurement of sulphotransferase activity in crude extracts is complicated by the presence of enzymes which degrade *P*-Ado-*PS* and thus effect the concentration of this nucleotide available as substrate. However, in terms of overall ability to sulphurylate oestrone, it would appear that the placenta is 3–4 times more active (per g wet weight of tissue) than the adrenal gland. Of the two placentae examined, the larger one was the more active.

TABLE I

OESTROGEN SULPHURYLATING ACTIVITY IN TISSUE EXTRACTS

Placenta 1: cotyledonary weight, 0.5 kg; foetal weight, 1.2 kg. Placenta 2: cotyledonary weight, 1.5 kg; foetal weight, 4.5 kg. Enzyme assay: standard conditions using 0.05 ml enzyme, 30 min incubation.

Tissue	Activity	
	nmoles oestrone sulphate per mg protein in 30 min	nmoles oestrone sulphate per g wet wt of tissue in 30 min
Adrenal cortex	3.5	88
Placenta 1	13.3	238
Placenta 2	20.2	360

Isolation and purification of the placental enzyme

The procedures developed for the purification of the adrenal enzyme [1] were also applied to the preparation of placental enzyme. The latter enzyme was found to behave identically to the adrenal enzyme through the various stages of purification.

Table II summarises the specific activity and yield during purification with 500 × *g* of placental tissue as starting material. The specific activities of three different placental preparations were 95 200 and 235 nmoles oestrone sulphate formed per mg protein in 20 min incubation. Thus, two of these preparations were more active than the usual adrenal preparations (specific activity 95–140) [1]. The isoenzyme pattern

TABLE II

PURIFICATION OF BOVINE PLACENTAL OESTROGEN SULPHOTRANSFERASE

Weight of placental tissue: 500 g. DEAE-cellulose I: column size 4 cm × 40 cm, stepwise phosphate buffer elution [1]. DEAE-cellulose II: column size 2.5 cm × 25 cm, elution with linear gradient, 0.02–0.1 M phosphate, pH 7.8 [1].

Stage	Protein (mg)	Specific activity (nmoles/mg in 20 min)	Units
Supernatant	8920	10	89 200
55–70% ammonium sulphate fraction	1930	26	50 100
DEAE-cellulose I (DE 11)	216	180	40 600
DEAE-cellulose II (DE 32)	140	200	28 000

as shown by acrylamide-gel electrophoresis consisted of four protein species [1] with the same electrophoretic mobilities as the four adrenal isoenzymes (Fig. 1). However, as mentioned in the previous paper [1], enzyme containing only three isoenzymes has been isolated from both adrenal and placental tissues obtained from individual animals.

Effect of enzyme concentration and time of incubation

Fig. 2a shows the increase in reaction rate with increasing protein concentration. The effect of time of incubation is shown in Fig. 2b.

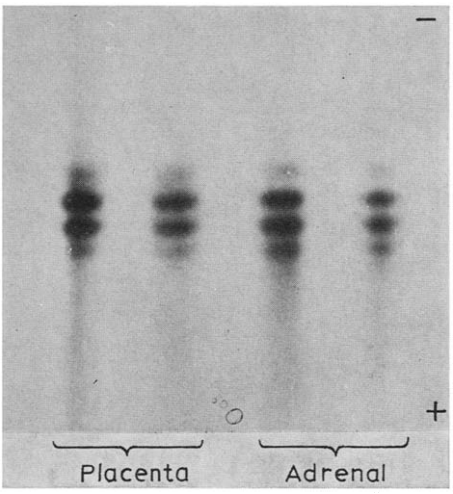

Fig. 1. Comparison of isoenzymes of oestrogen sulphotransferase derived from adrenals and placenta. Aliquots (20 and 40 μg protein) were run on slabs of acrylamide gel [1]. The adrenal preparation was obtained from some 50 adrenal glands whilst the placental preparation was derived from one animal.

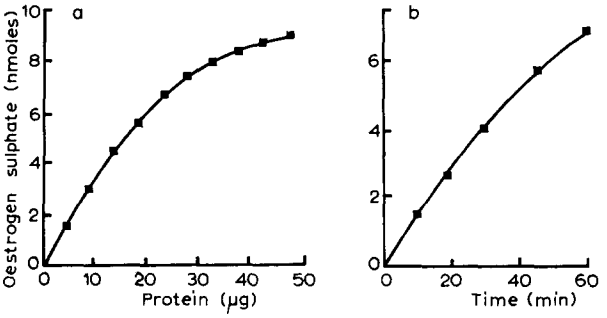

Fig. 2. a. Effect of enzyme concentration on reaction rate. b. Effect of time on product formation using 10 μg of enzyme. Standard conditions used at pH 7.5 (see Methods) but $MgCl_2$ omitted.

pH optimum

The pH optimum of the placental enzyme employing Tris–maleate–NaOH buffer was 6.2 (Fig. 3b). This value varied greatly from the optimum pH of 8.0 previously reported for the adrenal enzyme by Adams and Poulos [4] who used three different buffer systems to cover the pH range 6–10 (Tris–maleate–NaOH, Tris–HCl and glycine–NaOH). On redetermining the pH optimum of the adrenal enzyme with Tris–maleate–NaOH buffer alone, a value of 6.3 was obtained (Fig. 3a). A similar value was obtained with MES–glycylglycine–NaOH buffer (Fig. 3a). Thus the adrenal and placental enzymes behave similarly but it would appear that the actual composition of the buffer used has a marked effect on the pH optimum. It is interesting that the pH optimum for the partially-purified oestrogen sulphotransferase of guinea pig liver has been reported to be 6.2; the buffer system used in the determination was not however clearly stated [5]. Most of the studies presented in this communication were carried out in Tris–HCl buffer at pH 7.5. Velocity-substrate curves using Tris–HCl buffers at pH 7.0 and 8.0, respectively, retained their wave-like features [1] when

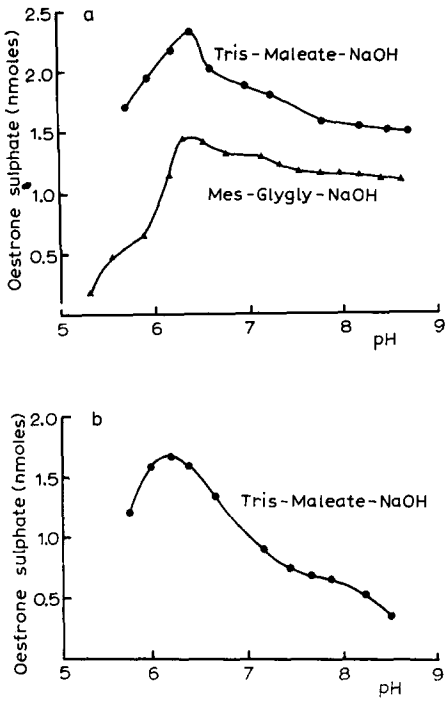

Fig. 3. Effect of pH on enzyme activity. a. Adrenal enzyme (25 μg) used with an incubation time of 30 min. b. Placental enzyme (10 μg) with an incubation time of 30 min. Buffer concentrations were 0.1 M.

oestrone was the variable substrate. Furthermore the positions of peaks and troughs were exactly duplicated at both pH values.

Specificity

Enzyme activity was measured with a number of different steroids at 0.04 mM concentration under standard assay conditions. The sulphurylation of 17β-oestradiol and oestriol, compared to oestrone as 1.0, were 1.02 and 1.10, respectively. The following compounds failed to act as substrate: dehydroepiandrosterone, etiocholanolone, 11-deoxycorticosterone, 17β-oestradiol-3-methyl ether, testosterone, pregnenolone, β-naphthylamine and *p*-nitrophenol. The specificity of the placental enzyme would therefore appear to be restricted to the 3-hydroxyl group of oestrogens as previously found for the adrenal enzyme [4].

Enzyme kinetics

The effect of varying oestrone, 17β-oestradiol and oestriol (0–60 μM) at a fixed and saturating concentration of *P*-Ado-*PS* is shown in Fig. 4. The wave-like kinetics typical of the adrenal enzyme [1] were again evident. The maximum velocity was greatest with oestriol but the shapes of the velocity–substrate curves were very similar for all three oestrogens, with peaks at approximately the same concentrations of oestrogen.

Kinetic studies were also carried out at a lower range of oestrone concen-

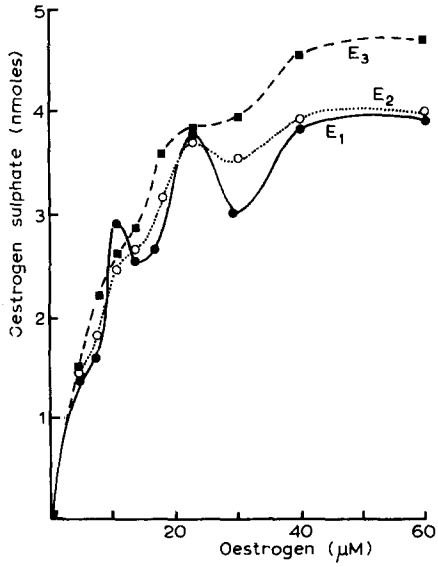

Fig. 4. Comparison of kinetics using three different oestrogens. E_1, oestrone; E_2, 17β-oestradiol; E_3, oestriol. Standard assay procedure employed (see Methods).

trations (0–8 μM). At these concentrations the curve was of the Michaelis–Menten type; the K_m for oestrone being 15 μM (Fig. 5a). When *P*-Ado-*PS* was varied and oestrone kept constant, a value of 37 μM was obtained for the K_m for *P*-Ado-*PS* (Fig. 5b). These two K_m values were identical with those determined for the adrenal enzyme [1].

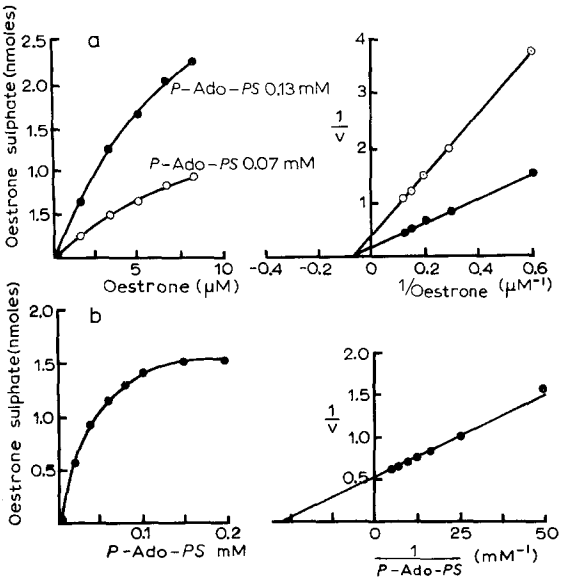

Fig. 5. a. Kinetics employing low oestrone concentrations at two levels of *P*-Ado-*PS*. b. Kinetics with *P*-Ado-*PS* as variable substrate. Oestrone concentration was 50 μM. Double reciprocal plots are shown on the right.

Thiol reagents

Cysteine and β-mercaptoethanol activated the enzyme at all concentrations of oestrone up to 30 μM. Dithiothreitol on the other hand activated only up to 5 μM oestrone and caused inhibition at concentrations greater than 5 μM. These results are thus very similar to those obtained with the adrenal enzyme [1].

Molecular weight

The molecular weight of the placental enzyme, determined by acrylamide-gel electrophoresis in the presence sodium dodecylsulphate as described in the previous paper [1], was 74 000–76 000.

Amino acid analysis

The amino acid compositions of two separate placental enzyme preparations, each containing a four-band pattern on gel electrophoresis, were determined. The results for the placental and adrenal preparations were similar enough to suggest that the two enzymes are identical, bearing in mind the amino acid differences already reported for the individual isoenzyme components [1].

Binding of endogenous oestrogens

It was previously demonstrated that oestrone was firmly bound to the adrenal enzyme. Thus upon incubation of the adrenal enzyme with *P*-Ado-$P^{35}S$ of high specific activity, liberation of oestrone [^{35}S]sulphate was detected. The latter was characterized by chromatography and co-crystallisation with authentic material [6]. The binding of oestrogens to the placental enzyme was also investigated in a similar manner. Enzyme was incubated with *P*-Ado-$P^{35}S$ of high specific activity and the products examined by chromatography in a solvent designed to separate the sulphates of the three classical oestrogens. A labelled derivative, with an R_F identical to that obtained in a parallel experiment using adrenal enzyme, and equal to that of oestrone sulphate, was formed.

DISCUSSION

The placentae of the guinea pig [7], the cow [2] and the sheep [8] all have the ability to sulphurylate oestrogens, but prior to the present studies, none of the sulphotransferases responsible have been isolated. Using the methods developed for the purification of bovine adrenal oestrogen sulphotransferase, bovine placental oestrogen sulphotransferase has been isolated in pure form. On the basis of the various criteria outlined in the results section, it can be concluded that placental enzyme is identical to that obtained from the adrenal. Placental preparations were on the average more active than adrenal preparations. Keeping in mind (i) the fact that isoenzymes of varying specific activities [1] are present, and (ii) the postulated model of the enzyme involving conformational species with different affinities for oestrogen [1], it would appear that any variations in specific activity is related firstly to the relative proportions of the isoenzymes and secondly to the relative proportions of the conformers.

Oestrone is the only oestrogen that has been identified in bovine adrenal tissue [9]. Although the ability of the bovine adrenal to sulphurylated oestrone was

demonstrated in 1963 [10], it is not known whether oestrone sulphate is secreted from the gland. Adams [6] demonstrated that oestrone was bound to bovine adrenal oestrogen sulphotransferase. Since no other oestrogen was detected this suggested that oestrone was the physiological substrate. This argument, if valid, would then also apply to the placental enzyme since only oestrone [^{35}S]sulphate was detected upon incubation of the purified enzyme with *P*-Ado-*P*^{35}*S*. It is perhaps of some significance also that oestrone showed exaggerated peaks and troughs in the kinetic experiments as compared to 17β-oestradiol and oestriol (Fig. 4).

In the pregnant cow, large amounts of oestrone, 17α-oestradiol and lesser amounts of 17β-oestradiol are found in the urine and it is claimed that the placenta is the principal source of this oestrogen [11]. The high sulphurylating ability of the bovine placenta stands in marked contrast to the human placenta which is virtually devoid of activity [12]. In the latter case, the placenta contains high levels of steroid sulphatases and in turn contrasts to the foetal compartment which has high steroid-sulphurylating ability. The situation with the bovine foetus is unknown but in the sheep, a phylogenetically related species, relatively high concentrations of oestrogen sulphates are found in foetal blood [13]. Pierrepoint et al. [8] have suggested that the placenta must be considered as a possible alternative to the foetal sheep liver as a source of these oestrogen conjugates. This was based on their observations that the placenta of the sheep converted androstenedione to oestrogen sulphates in relatively high yield in vitro.

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