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THE EFFECTS OF SPECIFIC INHIBITORS AND AN ANTISERUM OF 3β AND 17β-HYDROXYSTEROID DEHYDROGENASE ON STEROID UPTAKE IN *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

Uptake of testosterone by membrane vesicles and membrane bound 3β and 17β-hydroxysteroid dehydrogenase activity of *Pseudomonas testosteroni* were inhibited by diethylstilbestrol, estradiol-17α, hydroxymethylenesteroid and cyanoketosteroid. An antibody raised against purified 3β and 17β-hydroxysteroid dehydrogenase inhibited the activity of the membrane vesicle enzyme and uptake of testosterone. Accessibility of the antibody to the enzyme on the membrane vesicles indicated that the enzyme is located on the outer surface of the vesicle membrane.

INTRODUCTION

We have been characterizing steroid uptake by membrane vesicles prepared from *Pseudomonas testosteroni* [1–4], a gram-negative bacterium which can be induced to grow on some steroids as its sole carbon source [5–7]. The process is carrier-mediated [1] and the electron transport chain is required [4]. The involvement of the 3β and 17β-hydroxysteroid dehydrogenase in the uptake of steroids has been demonstrated by showing that both the uptake process and enzyme activity are identical with respect to (1) pH optima [1]; (2) cofactor requirements [1]; (3) substrate specificity [2]; (4) product formation [2]; and, (5) effect of certain sulfhydryl reagents [3].

Diethylstilbestrol*, estradiol-17α [7], cyanoketosteroid and hydroxymethylenesteroid [8,9] are potent inhibitors of the enzyme 3β and 17β-hydroxysteroid dehydrogenase purified from *Ps. testosteroni*. In this paper we report on the effect of these more specific inhibitors and of an anti-3β and 17β-hydroxysteroid dehydrogenase on the ability of membrane vesicles prepared from *Ps. testosteroni* to take up testosterone. These investigations confirm that the membrane bound 3β and 17β-hydroxysteroid dehydrogenase is involved in steroid uptake by membrane vesicles of *Ps. testosteroni*.

* Trivial and systematic nomenclature of steroids
Cyanoketosteroid = 2α-cyano-17β-hydroxy-4,4,17α-trimethylandrost-5-en-3-one; Diethylstilbestrol = 3,4-Bis (4-hydroxyphenyl)-3-hexene; Estradiol-17α = 1,3,5 (10)-estratriene-3,17α-diol; Hydroxymethylenesteroid = 17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one; Testosterone = 17β-hydroxy-4-androsten-3-one: Dihydrotestosterone = 17β-hydroxy-5β-androstane-3-one; Dehydroepiandrosterone = 3β-hydroxy-5-androsten-17-one.

EXPERIMENTAL PROCEDURES

Materials

Ps. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; Ready-Solv from Beckman Instruments, Inc., Fullerton, California; unlabelled testosterone from Steraloids, Pawling, New York; NAD^+ from Schwarz-Mann, Orangeburg, New York; [1,2-^{3}H]-testosterone and [7-^{3}H]-testosterone from New England Nuclear Corporation, Boston, Massachusetts.

Methods

Media used for growth and conditions for induction of binding activity have been described [10]. Membrane vesicles were prepared after spheroplast formation using the lysozyme–EDTA method, as described by Kaback [11]. Whole cells were harvested by centrifugation at 12,000 *g* for 10 min. and washed once by resuspension in 10 mM Tris–HCl, pH 8.0, at 0°C before being used in experiments.

Uptake studies. Testosterone uptake was assayed as previously described [1]. Reaction mixtures for transport studies contained in 0.2 ml: 50 μmol Tris–HCl, pH 9.0, 200 nmol NAD^+, approximately 170 pmol of labelled testosterone (33×10^5 d.p.m./nmol), and 2 to 10 μg of membrane protein. The reaction mixture was incubated at 25°C for 2 min.

Enzyme assay. The assay for 3β and 17β-hydroxysteroid dehydrogenase was performed as described by Talalay [12]. One unit of enzyme was defined as

Table 1. Inhibition of membrane vesicle 3β and 17β-hydroxysteroid dehydrogenase

Inhibitor (33 μM)	3β and 17β-Hydroxysteroid dehydrogenase activity (nmol NADH/min/mg protein)	(% control)
None	6.27	100
Diethylstilbestrol	2.26	36
Estradiol-17α	2.37	37
Cyanoketosteroid	0.00	0
Hydroxymethylenesteroid	4.70	75

Note: Enzyme assays were performed as described in Methods. The inhibitor was added in 0.05 ml methanol. The control also contained 0.05 ml methanol. Duplicate samples were assayed.

reduction of 0.48 nmol NAD^+ under the specified conditions.

Protein was determined by the method of Lowry *et al.*[13].

Antibody studies. The antibody yielded a single, continuous and sharp precipitin band in Ouchterlony double diffusion tests against both pure enzyme and a crude extract of induced cells [14]. Preimmune serum did not inhibit or cross-react with 3β and 17β-hydroxysteroid dehydrogenase activity. Unless stated otherwise, before performing steroid uptake or 3β and 17β-hydroxysteroid dehydrogenase assays, approximately 400 μg membrane vesicle protein with varying amounts of antibody in 5 mM Hepes buffer in a final volume of 0.05 ml were incubated at room temperature for 30 min. and then at 2°C for 20 h. Antiserum which had been placed in a boiling water bath for 10 min. was used as a control. For enzyme assays, the entire contents of a tube were used. For uptake studies, the suspension was diluted 30-fold and 0.1 ml was used.

RESULTS

Inhibition of the testosterone uptake process by inhibitors of 3β and 17β-hydroxysteroid dehydrogenase

Table 1 shows that diethylstilbestrol, estradiol-17α, cyanoketosteroid and hydroxymethylenesteroid, known inhibitors of the purified 3β and 17β-hydroxysteroid dehydrogenase from extracts of *Ps. testosteroni* [7–9], also cause potent inhibition of the enzyme bound to membrane vesicles prepared from this organism. At a concentration of 33 μM these four compounds caused marked inhibition of the membrane vesicle 3β and 17β-hydroxysteroid dehydrogenase.

The effect of these four inhibitors on testosterone uptake was investigated by measuring uptake at increasing concentrations of testosterone in the presence of two concentrations of the inhibitor and comparing these results to uptake in the absence of inhibitor. Lineweaver–Burk plots for each inhibitor were drawn. Figures 1 and 2 show that each of these com-

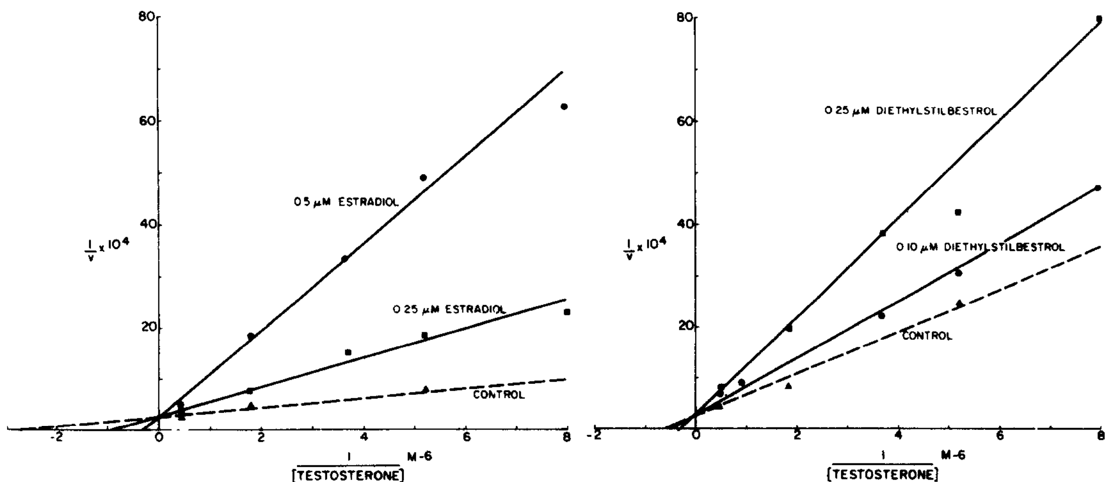

Fig. 1. The effect of estradiol-17α and diethylstilbestrol on uptake of testosterone by membrane vesicles of *Ps. testosteroni*. Uptake of testosterone was determined as described in Methods in the presence of increasing amounts of testosterone at two concentrations of (a) estradiol-17α and (b) diethylstilbestrol. *V* is in pmol/min/mg protein. Uptake at each concentration of testosterone is the mean of four experiments.

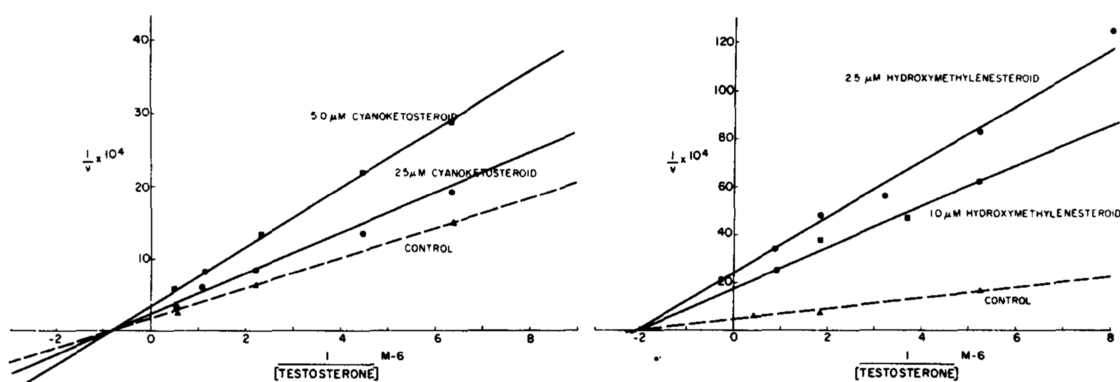

Fig. 2. The effect of cyanoketosteroid and hydroxymethylenesteroid on uptake of testosterone by membrane vesicles of *Ps. testosteroni*. Uptake of testosterone was determined as described in Methods in the presence of increasing amounts of testosterone at two concentrations of (a) cyanoketosteroid and (b) hydroxymethylenesteroid. *V* is in pmol/min/mg protein. Uptake at each concentration of testosterone is the mean of four experiments.

pounds inhibits testosterone uptake by vesicles. Different preparations of membrane vesicles were used for each inhibitor study. The apparent K_M was 1.67 μM in the cyanoketosteroid and diethylstilbestrol study, 0.50 μM in the hydroxymethylenesteroid experiment and 0.39 μM in the estradiol experiment. Figures 1(a) and 1(b) show that diethylstilbestrol and estradiol-17α inhibit testosterone uptake in a competitive manner, while Figs 3a and 3b show that cyanoketosteroid and hydroxymethylenesteroid inhibit steroid uptake in a non-competitive manner. Neville and Engel[9] found that cyanoketosteroid and hydroxymethylenesteroid inhibited the more purified 3β and 17β-hydroxysteroid dehydrogenase in a non-competitive manner.

Inhibition of membrane vesicle 3β and 17β-hydroxysteroid dehydrogenase and testosterone uptake by an anti-3β and 17β-hydroxysteroid dehydrogenase

Table 2 shows that the anti-3β and 17β-hydroxysteroid dehydrogenase inhibited both the membrane vesicle dehydrogenase and testosterone uptake, while boiled antibody had no effect on either process. In addition, it was found that an overnight incubation of the membrane vesicle was not necessary for inhibition of the enzyme activity or the testosterone uptake process.

In another experiment membrane vesicle testosterone uptake was measured in the presence of increasing amounts of antibody. Fig. 3 shows that it is possible to completely inhibit the uptake process.

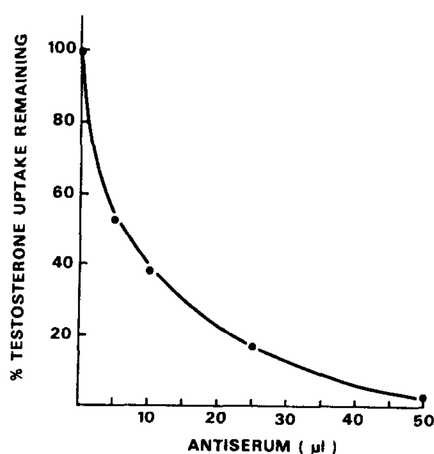

Fig. 3. Inhibition of testosterone uptake by membrane vesicles with antiserum to 3β and 17β-hydroxysteroid dehydrogenase. Membrane vesicles were incubated with increasing amounts of anti-3β and 17β-hydroxysteroid dehydrogenase before testosterone uptake was determined as described in Methods.

DISCUSSION

We have previously presented data that implicated the membrane vesicle-associated 3β and 17β-hydroxysteroid dehydrogenase activity of *Ps. testosteroni* with uptake of steroids by these vesicles. Both processes operate maximally between pH 8 and 9 [1]; require NAD^+ [1]; use testosterone, dihydrotestosterone or dehydroepiandrosterone to produce androstenedione, androstanedione or androstenedione respectively [2]. In addition, both processes are inhibited in a similar manner by the sufhydryl reagents N-ethylmaleimide, *p*-hydroxymercuribenzoate, $ZnSO_4$ and $CuSO_4$ [3]. While these results provided•convincing arguments for the involvement of the 3β and 17β-hydroxysteroid dehydrogenase in the uptake process, the experiments reported here provide conclusive evidence.

Known specific inhibitors of the enzyme activity, diethylstilbestrol, estradiol-17α, cyanoketosteroid and hydroxymethylenesteroid, also inhibit steroid uptake by the membrane vesicles. Cyanoketosteroid and hydroxymethylenesteroid, which have been shown to in-

Table 2. Effect of antibody to 3β and 17β-hydroxysteroid dehydrogenase on membrane vesicle 3β and 17β-hydroxysteroid dehydrogenase and on testosterone uptake

	3β and 17β-hydroxysteroid dehydrogenase activity		Uptake of testosterone	
Amount of antibody	(nmol NADH/min/mg protein)	(% Control)	(nmol/min/mg) protein	(% Control)
A. 0.000 ml	5.43	100	1.59	100
B. 0.005 ml	2.87	53	0.94	59
C. 0.005 ml Boiled	5.43	100	1.59	100
D. 0.005 ml No preincubation	2.68	49	0.78	49

Note: Assays of 3β and 17β-hydroxysteroid dehydrogenase and testosterone uptake in the presence of antibody were performed as discussed in Methods, except in experiment D where preincubation at room temperature or at 2 C was not done.

hibit the enzyme activity in a non-competitive manner [9] inhibit the uptake process non-competitively.

Finally, rabbit anti-3β and 17β-hydroxysteroid dehydrogenase causes inhibition of steroid uptake by membrane vesicles. The antibody appears to act at the catalytic site of the membrane-bound enzyme, thus causing inhibition of the dehydrogenation activity and simultaneous inhibition of the steroid uptake activity. The ability of the antibody to inhibit the vesicle enzyme and the short time necessary for the inhibition to take place indicated that the dehydrogenase is easily accessible to the antibody and is probably located on the outside of the vesicle membrane [15, 16].

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