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PERIPLASMIC STEROID-BINDING PROTEINS AND STEROID TRANSFORMING ENZYMES OF *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

Exposure of induced *Pseudomonas testosteroni* to osmotic shock resulted in the release of androgen and estrogen-binding proteins, 5-ene-3-ketosteroid isomerase and (3 and 17)β-hydroxysteroid dehydrogenase activities, and variable amounts of 3α-hydroxysteroid dehydrogenase, 1-ene-dehydrogenase and 4-ene-5α-dehydrogenase activities. When binding of C19 and C21 steroids to induced bacteria and to periplasmic proteins was examined, binding correlated with the ability of the steroids to support growth of this organism. Except for estradiol-17β, steroids incapable of supporting growth, such as aldosterone, cortisol, and corticosterone, were not bound. In the case of C19 steroids, the bound form of the steroid was androstanedione and/or androstenedione. On the other hand, progesterone and 17α-hydroxyprogesterone were bound in the form in which they were added. Displacement studies with unlabeled steroids indicated the presence of two steroid receptors, one which bound C19 and C21 steroids, and another which bound estradiol-17β.

INTRODUCTION

Recent studies from our laboratory have indicated that during adaptive growth of *Pseudomonas testosteroni* on testosterone,* a steroid-binding activity and a steroid transport process are induced [1–3]. The binding activity, which can be released from induced bacteria by osmotic shock [1], possesses characteristics of a protein molecule. The exact role of the receptor protein is under investigation, but as with other bacterial periplasmic proteins, it is postulated to be involved in some aspect of steroid transport through the cell membrane.

P. testosteroni grows on several C19 and C21 steroids as a sole carbon source [4, 5]. We have examined the ability of several of these steroids to induce binding activity. In addition to testosterone, other C19 steroids such as androstenedione, androsterone, 5α- and 5β-dihydrotestosterone and dehydroepiandrosterone, and the C21 steroid, progesterone, all induced testosterone binding activity [2]. Furthermore, the receptor protein induced by growth on testosterone bound androstenedione as well as C21 steroids, such as progesterone and 17α-hydroxyprogesterone [2]. In an effort to define the steroid transport process in this organism, we have asked the following questions. Assuming that there is a unique steroid transport system, do all C19 and C21 steroids share a common system or is there a specific system for each steroid? If there is a common system, what is the mechanism by which various steroids are accommodated? To begin to answer these questions we have examined the nature of the binding of various C18, C19 and C21 steroids to intact organisms and to periplasmic proteins released by osmotic shock.

* Trivial and systematic nomenclature of steroids: androsterone, 3α-hydroxy-5α-androstan-17-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; androstenedione, 4-androstene-3,17-dione; 5α-androstanedione, 5α-androstan-3,17-dione; 5β-androstanedione, 5β-androstan-3,17-dione; testosterone, 17β-hydroxy-4-androsten-3-one; epitestosterone, 17α-hydroxy-4-androsten-3-one; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; aldosterone, 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al; progesterone, 4-pregnene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; estradiol-17β, 1,3,5(10)-estratriene-3,17β-diol.

EXPERIMENTAL PROCEDURES

Materials

Unlabeled steroids were obtained from Mann Research Laboratories, New York, New York and Steraloids, Inc., Pawling, New York; Dextran T70 from Pharmacia, Uppsala, Sweden; and Kieselgel N from Mackerey, Nagel and Company, Germany. The following radioactive steroids were all purchased from

New England Nuclear Corporation, Boston, Massachusetts: [1,2-^{3}H]-androsterone (53 Ci/mmol), [1,2-^{3}H]-testosterone (45 Ci/mmol), [1,2-^{3}H]-5α-dihydrotestosterone (44 Ci/mmol), [1,2-^{3}H]-androstenedione (48 Ci/mmol), [1,2-^{3}H]-epitestosterone (55 Ci/mmol), [1,2-^{3}H]-aldosterone (50 Ci/mmol), [1,2-^{3}H]-cortisol (43 Ci/mmol), [1,2-^{3}H]-progesterone (50·3 Ci/mmol), [1,2-^{3}H]-17α-hydroxyprogesterone (49·2 Ci/mmol), [7-^{3}H]-testosterone (25 Ci/mmol), [7-^{3}H]-17α-hydroxyprogesterone (10 Ci/mmol), [7-^{3}H]-progesterone (20 Ci/mmol), [7-^{3}H]-dehydroepiandrosterone (21 Ci/mmol), [6,7-^{3}H]-estradiol-17β (46·6 Ci/mmol), [1,2-^{3}H]-deoxycorticosterone (40 Ci/mmol), [1,2-^{3}H]-corticosterone (44 Ci/mmol). [1,2-^{3}H]-5α-androstanedione (53 Ci/mmol) was prepared from [1,2-^{3}H]-androsterone by CrO_3 oxidation [6].

Methods

The conditions and medium used for growth of *P. testosteroni*, and the methods used for induction of binding activity by testosterone, preparation of osmotic shock protein, assay for binding by intact induced bacteria and osmotic shock protein and steroid degradative activity, and sucrose density gradient centrifugation have been described [1, 2].Cultures induced for 40 h were used in all studies.

Enzyme assays. The assays for 3α- and (3 and 17)β-hydroxysteroid dehydrogenases were performed as described by Talalay [7]. The reaction mixture contained in 3·0 ml, 100 μmol of sodium pyrophosphate buffer, pH 8·9, 0·5 μmol of NAD^+, 0·1 μmol of androsterone (3α enzyme) or testosterone (3 and 17β enzyme) in 0·02 ml of redistilled ethanol, and a suitable amount of enzyme. The reaction was carried out at 27°C and absorbance at 340 nm was measured at 15 s intervals. Reduction of NAD^+ was determined by the change of absorbance at this wavelength. One unit of enzyme was defined as reduction of 0·48 nmol of NAD^+ under the specified conditions.

The assay for 5-ene-3-ketosteroid isomerase was performed as described by Kawahara [8]. The reaction mixture contained in 3·0 ml, 100 μmol of phosphate buffer, pH 7·0, 0·175 μmol of 5-androstene-3,17-dione in 0·05 ml of methanol, and a suitable amount of enzyme. The reaction was carried out at 27°C and the optical density measured at 248 nm at 15 s intervals. One unit of enzyme was defined as that amount which isomerized 1 μmol of steroid per min under these conditions.

The 1-ene-dehydrogenase activity was determined by measuring conversion of [1,2-^{3}H]-steroid to ^3H_2O by a method previously described [2]. The assays for 4-ene-5α- and 4-ene-5β-dehydrogenases were performed as described by Levy, using U.V.-absorption measurement of the products [9].

Analysis of steroid in medium and steroid bound to osmotic shock protein and to induced bacteria

Osmotic shock protein (3·4 mg), in a total volume of 5 ml, was equilibrated with 15 ml of dialyzing buffer containing 50 mM Tris–HCl, pH 9·0, 7 nM tritiated steroids, 5 mM EDTA and 0·5 mM dithiothreitol, at 4°C, for 20 h. At the end of dialysis, 1·5 ml of dextran-coated charcoal (2·5% neutral Norit–0·25% Dextran T70) was added to the dialysate to absorb the free steroid and the charcoal subsequently removed by centrifugation at 10,000 rev./min for 10 min at 4°C in an International B20 refrigerated centrifuge. The remaining steroid–protein complex and the dialysis medium were then extracted three times with dichloromethane. The extracts were evaporated to dryness under nitrogen at 45°C.

For binding studies by intact bacteria, reaction mixtures contained in 10 ml, 3 ml of induced bacteria, 100 mM Tris HCl, pH 9·0, and 4 nmol of labeled steroids. The mixture was incubated at 4 C for 10 min and the bacterial cells were harvested by centrifugation at 10,000 rev./min for 10 min at 4°C. The pellet was resuspended in Tris buffer containing 400 nmol of unlabeled steroids and following displacement of the bound radioactive steroids by unlabeled steroids, the bacteria was removed by centrifugation. The supernatants from both centrifugations were extracted three times with dichloromethane and three times with ethyl acetate and the extracts were evaporated to dryness under nitrogen at 45°C.

The dried extracts were analyzed by several thin-layer chromatography systems as well as by gas–liquid chromatography. Thin-layer chromatography was performed on 0·4 mm silica gel-coated plates using the systems benzene absolute ethanol 19:1 v/v, n-hexane ethyl acetate 75:25 v/v, chloroform–absolute ethanol 19:1 v/v, and hexane–acetone, 6:4 [10]. Reference standards were located by spraying with a 1% (v/v) solution of anisaldehyde in a 2% (v/v) solution of concentrated sulfuric acid in glacial acetic acid and heating to 100°C [10]. One centimeter portions were scraped and assayed for radioactivity using Bray's solution [11]. Gas–liquid chromatography was carried out on extracts with a model 810 F and M chromatograph equipped with a stream splitter. The column was 5% OV-210 (8 ft × 4 mm i.d.); carrier gas, helium; flow rate, 60 ml/min; column temperature, 220°C. The effluent was assayed for radioactivity in a liquid scintillation spectrometer using Bray's reagent.

RESULTS

Binding of various steroids by induced organisms and by osmotic shock protein

We have previously reported that during growth of *P. testosteroni* on testosterone a steroid-binding activity, in addition to steroid degradative enzymes, is induced [2]. This steroid binding activity could be released by exposure of induced organisms to osmotic shock [1]. The ability of induced organisms and osmotic shock proteins prepared from such cultures to bind C19, C21 and C18 steroids and to degrade steroids have been examined. In general, binding activity of both intact organisms and periplasmic protein paralleled the capability for steroid degradation. All of the C19 and C21 steroids which were shown to be degraded were bound by both intact organisms and osmotic shock protein (Table 1). The C21 steroids which were not degraded, such as aldosterone, corticosterone and cortisol, were not bound. Estradiol was the only steroid which was not degraded but which was bound to a significant degree.

Displacement from binding sites by unlabeled steroids

In order to determine whether the steroids were bound to a single protein or to separate receptor proteins, displacement studies were performed. For both intact organism and osmotic shock protein, steroids which bound, displaced labeled testosterone or androstanedione from binding sites except for estradiol-17β (Table 2). Steroids which were not bound, such as aldosterone, corticosterone, and cortisol, did not affect the binding of radioactive androstanedione or testosterone. Neither unlabeled testosterone nor 5α-androstanedione inhibited [^{3}H]-estradiol-17β binding by osmotic shock protein (Table 3). The inability of estradiol-17β to displace androstanedione or testosterone, and of the latter two steroids to displace [^{3}H]-estradiol-17β, indicated a binding site for estradiol-17β which was distinct from that which bound the C19 and C21 steroids.

Table 1. Binding of C19, C18 and C21 steroids by induced *P. testosteroni* and by osmotic shock protein

Steroid	Steroid degraded	Steroid bound	
	By intact organism (pmol/10 min)	By intact organism (pmol)	By osmotic shock protein (pmol/mg protein)
C19 steroids			
[1,2-^{3}H]-androstanedione	58·8	5·0	24·6
[1,2-^{3}H]-dihydrotestosterone	42·0	4·9	15·0
[1,2-^{3}H]-androsterone	60·3	4·3	15·6
[7-^{3}H]-dehydroepiandrosterone	32·3	4·3	6·9
[1,2-^{3}H]-androstenedione	53·2	3·1	12·3
[1,2-^{3}H]-testosterone	46·6	2·5	11·5
[1,2-^{3}H]-epitestosterone	42·6	1·3	3·0
C21 steroids			
17α-[7-^{3}H]-hydroxyprogesterone	44·4	7·6	17·2
[7-^{3}H]-progesterone	32·9	4·2	14·0
[1,2-^{3}H]-deoxycorticosterone	—	—	5·2
[1,2-^{3}H]-aldosterone	2·3	0·2	—
[1,2-^{3}H]-corticosterone	0	—	0
[1,2-^{3}H]-cortisol	0·5	0·5	0
C18 steroids			
[6.7-^{3}H]-estradiol-17β	0	1·3	7·4

Note 1. Reaction mixtures for binding by intact bacteria contained in 1 ml 50 mM Tris, pH 9·0, 0·5 ml of induced or uninduced bacteria washed once with Tris buffer and 40 pmol of radioactive steroid (approx. 3×10^6 c.p.m./nmol). Binding activity was determined at 4°C after harvesting the cells by centrifugation. The results shown represent the difference between induced and uninduced bacteria.

Note 2. Binding of steroids by osmotic shock protein was determined by equilibrium dialysis. Osmotic shock protein, in a total volume of 1 ml, was equilibrated with 15 ml of dialyzing buffer containing 5 mM EDTA, 0·5 mM dithiothreitol, 7 nM tritiated steroids and 50 mM Tris–HCl, pH 9·0 at 4°C for 20 h. Tritium present inside and outside the bag was determined in a liquid scintillation spectrometer.

Note 3. The reaction mixture for degradative activity contained in 1 ml, 50 mM Tris–HCl, pH 9·0, 75 pmol of labeled steroid and 0·3 ml of uninduced or induced bacteria. The reaction mixture was incubated at 30°C for 10 min and conversion of the tritium label to ^3H_2O was determined as previously described [2]. The figures represent the difference between induced and uninduced cultures.

Table 2. Effect of unlabeled steroids on binding of [^{3}H]-androstanedione to intact bacteria and [7-^{3}H]-testosterone to osmotic shock protein

Steroid	Concentration of unlabeled steroids required to inhibit binding by 50%	
	Intact bacteria (nM)	Osmotic shock protein (nM)
Testosterone		6·2
5α-Androstanedione	34	5·4
5β-Androstanedione	43	5·5
Androsterone	43	4·8
5α-Dihydrotestosterone	89	5·5
5β-Dihydrotestosterone	55	
Androstenedione	29	5·9
Epitestosterone	140	20·0
Dehydroepiandrosterone	83	40·0
17α-Hydroxyprogesterone	33	11·1
Progesterone	43	3·2
Deoxycorticosterone	71	
No displacement with	Aldosterone Corticosterone Cortisol Estradiol-17β	 Cortisol Estradiol-17β

Note 1. The reaction mixture for binding to bacteria contained 0·5 ml induced bacteria, 50 mM Tris–HCl, pH 9·0, 5 pmol of [1,2-^{3}H]-androstanedione (3×10^7 c.p.m./nmol) and increasing concentrations of unlabeled steroid up to 350 mM. The reaction mixture was allowed to stand at 4°C for 1 min and the radioactivity bound to bacteria was determined after centrifugation. The amount of [3H]-androstanedione bound in the absence of unlabeled steroid represents 100% in these calculations.

Note 2. The binding of [7-^{3}H]-testosterone to osmotic shock protein was determined as outlined in Table 1. The dialysis medium contained 50 mM Tris–HCl, pH 9·0, 5 mM EDTA, 0·5 mM dithiothreitol, 2·6 nM [7-^{3}H]-testosterone ($1·9 \times 10^7$ c.p.m./nmol) and unlabeled steroid at various concentrations. The amount of [^{3}H]-testosterone bound in the absence of unlabeled steroid represents 100% in these calculations.

Analysis of steroid bound by osmotic shock protein

The displacement studies with unlabeled steroids indicated that the various steroids, with the exception of estradiol-17β, were bound to a single binding site, thus suggesting either a lack of rigid structural specificity for binding, or enzymatic transformation of the steroids prior to binding. The nature of the bound form of the steroid was therefore investigated. With labeled testosterone, androstenedione, or dehydroepiandrosterone, the steroid bound to osmotic shock protein was found to be androstenedione (Table 4). The major portion of the steroid recovered in the dialysis medium was also androstenedione. When androstanedione, dihydrotestosterone and androsterone were used for the binding reaction, about one-third of the bound steroid was in the form of androstenedione. One-third was recovered as 5α-androstanedione, while the other third was comprised of polar metabolites. The dialysis medium contained the same proportion of metabolites, although in the case of androsterone, there appeared to be a greater amount of polar metabolites in the medium. Except for androstenedione, androstanedione, and to a lesser extent androsterone, the labeled

Table 3. Effect of testosterone and androstanedione on [6,7-^{3}H]-estradiol-17β binding by osmotic shock protein

Unlabeled steroid	Concentration (nM)	[^{3}H]-estradiol bound (pmol/mg protein)
None		2·4
Testosterone	4·6	2·4
	11·5	2·5
5α Androstanedione	4·6	2·2
	11·5	2·5

Note 1. Osmotic shock protein was dialyzed against 1·4 nM [6,7-^{3}H]-estradiol-17β ($4·5 \times 10^7$ c.p.m. nmol) with either unlabeled testosterone or 5α-androstanedione added in the concentration indicated.

Table 4. Analysis of steroid in dialysis medium and steroid bound to osmotic shock protein

Labeled steroid added to reaction mixture	Source	Steroids recovered (% of total radioactivity in sample)			
		Androstenedione	5α-Androstanedione	Androsterone	Polar metabolites
[7-^{3}H]-testosterone	Bound	100			
	Medium	91·2			6·4
[1,2-^{3}H]-androstenedione	Bound	100			
	Medium	98·3			1·1
[1,2-^{3}H]-dehydroepiandrosterone	Bound	90·2	1·9		7·8
	Medium	90·9	1·3		5·2
[1,2-^{3}H]-5α-dihydrotestosterone	Bound	37·3	22·9		36·4
	Medium	24·1	37·6		33·6
[1,2-^{3}H]-5α-androstanedione	Bound	41·7	35·1		22·8
	Medium	39·3	31·7		27·3
[1,2-^{3}H]-androsterone	Bound	34·0	45·4		18·8
	Medium	17·4	6·7	6·4	67·6

Note 1. The experiment was performed and steroids analyzed as described in the text. The figures represent data obtained from t.l.c. using the system n-hexane–ethyl acetate 75:25. Similar results were obtained with the other thin-layer chromatography solvent systems as well as by gas–liquid chromatography.

C19 steroids were not recovered in the dialysis medium in the forms in which they were added.

When progesterone or 17α-hydroxyprogesterone was added for binding studies, the steroid bound to osmotic shock protein was recovered mainly as unaltered progesterone or 17α-hydroxyprogesterone, respectively. Similarly, the steroid in the medium remained unaltered.

The nature of steroid bound by intact induced organisms was also investigated. The findings were essentially similar to those observed with isolated periplasmic proteins. When labeled testosterone, androstenedione, dihydrotestosterone or androsterone was added, the bound form of the steroid was predominantly androstenedione (Table 5). Small amounts of testosterone were also bound. After 10 min of incubation at 4°C most of the labeled testosterone and dihydrotestosterone in the medium was converted to androstenedione. Most of the androsterone remained unaltered in the medium although significant amounts of androstenedione were also detected.

Sucrose density gradient analysis of osmotic shock protein–steroid complex

When shock protein–testosterone complex was analyzed by sucrose density gradient centrifugation, a single peak of bound hormone was obtained with a sedimentation constant of approximately 4S [1]. The osmotic shock protein labeled with radioactive androstenedione, androstanedione, androsterone, dihydrotestosterone, dehydroepiandrosterone, progesterone, 17α-hydroxyprogesterone and estradiol-17β also appeared as a single radioactive peak in each case on sucrose density gradient centrifugation (Fig. 1). The sedimentation coefficient of each steroid–protein complex, including the estradiol–protein complex, was ap-

Table 5. Analysis of steroid in medium and bound to induced bacteria

Labeled steroids added to reaction mixture	Source	Steroids recovered (% of total radioactivity in sample)			
		Androstenedione	Testosterone	5α-Dihydro-testosterone	Androsterone
[1,2-^{3}H]-testosterone	Bound	40·7	8·7		
	Medium	64·2	15·6		
[1,2-^{3}H]-androstenedione	Bound	78·8			
	Medium	97·7			
[1,2-^{3}H]-5α-dihydrotestosterone	Bound	59·0	12·4	2·8	
	Medium	67·8	8·4	8·2	
[1,2-^{3}H]-androsterone	Bound	41·5	2·2		24·4
	Medium	17·4	0·5		67·8

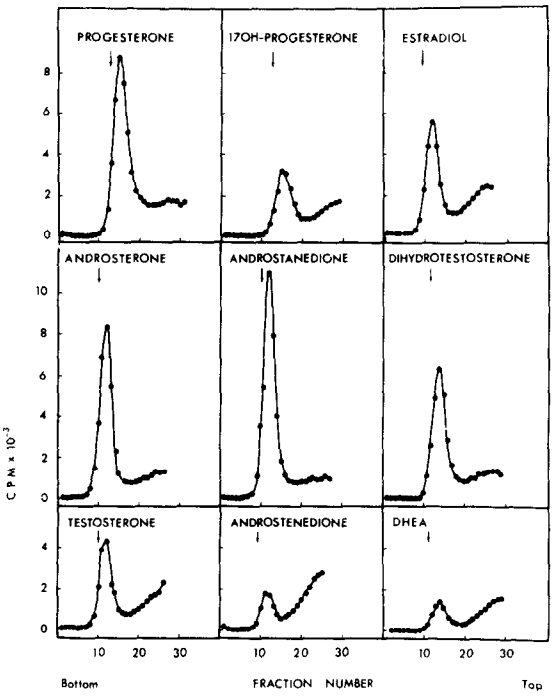

Fig. 1. Sucrose density gradient centrifugation of osmotic shock protein labeled with various tritiated steroids. Osmotic shock protein, in a total volume of 1 ml, was equilibrated with 15 ml of a buffer containing 50 mM Tris–HCl, pH 9·0, 7 nM tritiated steroids, 5 mM EDTA, and 0·5 mM dithiothreitol, at 4°C for 20 h. A 0·2 ml sample of the dialysate was layered on a linear 5–20% sucrose gradient containing 10 mM Tris–50 mM NaCl, pH 7·2. The gradients were centrifuged at 40,000 rev./min for 15 h in a SW 56 rotor at 4°C in a Beckman Ultracentrifuge Model L2-65B. The gradients were then fractionated by piercing the bottom of the tube and collecting 5 drops per vial. Bovine serum albumin (↓) was added as a marker to the samples before centrifugation.

proximately 4 S in 0·05 M NaCl. In the case of androsterone, androstanedione and dihydrotestosterone, although the bound form included both androstenedione and androstanedione (Table 4), only a single peak of bound radioactivity was observed.

Enzyme activity of osmotic shock fluid

The finding that 5α-androstanedione and/or androstenedione were the predominant C19 steroids bound to receptor proteins suggested that the periplasmic fluid contained significant enzyme activity capable of transforming steroids. The six enzymes known to be induced by steroids were therefore examined in periplasmic fluid released by osmotic shock. Two enzymes, 5-ene-3-ketosteroid isomerase and (3 and 17)β-hydroxysteroid dehydrogenase, were present in significant amounts whereas only minimal and variable levels of 3α-hydroxysteroid dehydrogenase, 4-ene-5α-dehydrogenase and 1-ene-dehydrogenase were detected (Table 6). 4-ene-5β-Dehydrogenase activity was not detected in any of the experiments.

DISCUSSION

A number of enzymes localized in the periplasmic space are known to be released by osmotic shock treatment of Gram-negative bacteria [12]. The preparation of osmotic shock fluid in the present study contained a number of steroid-transforming enzymes which have been previously described in induced cultures of *P. testosteroni* [13, 14]. These enzyme activities included (3 and 17)β-hydroxysteroid dehydrogenase, 3α-hydroxysteroid dehydrogenase, 5-ene-3-ketosteroid isomerase, and 4-ene-5α-dehydrogenase. In addition to these enzyme activities, at least two steroid-binding proteins appeared to be released by osmotic shock. One protein appeared to bind C19 and C21 steroids, while the second protein bound estradiol, but not C19 or C21 steroids.

Analysis of the C19 steroid bound to induced bacteria or to osmotic shock protein indicated that the

Table 6. Periplasmic enzymes of induced *Pseudomonas testosteroni*

Enzyme	Enzyme activity (pmol/min/mg protein)			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
(3 and 17)β-Hydroxysteroid dehydrogenase	$58{\cdot}1 \times 10^3$	$160{\cdot}9 \times 10^3$	$132{\cdot}2 \times 10^3$	$11{\cdot}7 \times 10^3$
3α-Hydroxysteroid dehydrogenase	0	$3{\cdot}8 \times 10^3$	0	$0{\cdot}9 \times 10^3$
5-ene-3-Ketosteroid isomerase	$38{\cdot}2 \times 10^6$	$16{\cdot}9 \times 10^6$	$11{\cdot}7 \times 10^6$	$9{\cdot}2 \times 10^6$
1-ene-Dehydrogenase	0	0	0·62	0·05
4-ene-5α-Dehydrogenase	0	0	1720	0
4-ene-5β-Dehydrogenase	0	0	0	0

Note 1. 3α- And (3 and 17)β-hydroxysteroid dehydrogenase activities were measured as pmols of NAD^+ reduced per min per mg enzyme protein. All other enzyme activities were measured as pmol of steroid metabolized per min/mg enzyme protein.

bound form was androstanedione and/or androstenedione regardless of the nature of the starting compound. Androstanedione and androstenedione were bound to the same protein as judged by their ability to displace one another from binding sites. Displacement studies also suggested that progesterone and 17α-hydroxyprogesterone were bound to the same receptor, although they were not transformed to androstenedione.

The present data can be interpreted to indicate that the binding protein had a high structural specificity for androstenedione and androstanedione among C19 steroids. On the other hand, periplasmic enzymes are able to convert various C19 steroids to androstenedione and androstanedione (Fig. 2), and such transformations may be so efficient that steroids were not available for binding in the form in which they were added. Purification of the binding protein will be necessary in order to determine binding specificity.

The functional significance of the periplasmic binding protein was indicated by the finding that, in general, only those steroids capable of supporting growth of this organism were bound. Steroids such as aldosterone, corticosterone and cortisol, which are not metabolized [4, 5], were not bound by either intact organism or osmotic shock protein. Estradiol, a steroid which is not capable of being oxidized by this organism [4, 5], was bound to osmotic shock protein and induced bacteria. However, it was found that this compound was bound to a receptor distinct from that for the other steroids. It would be of interest to determine the functional significance of estradiol binding.

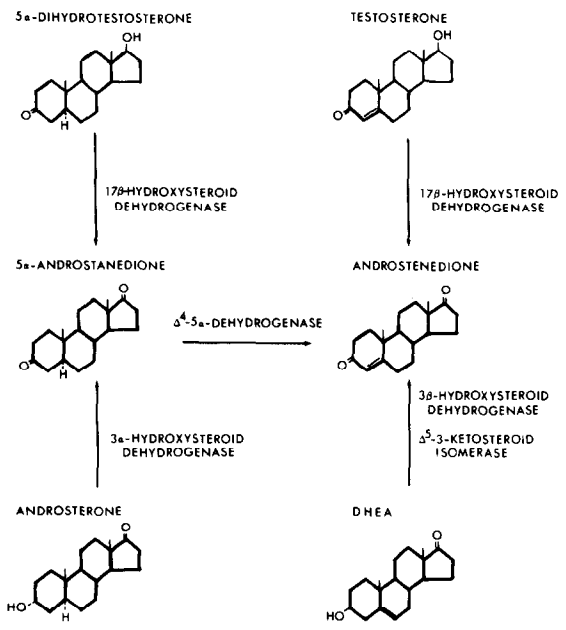

Fig. 2.

The role of steroid-transforming enzymes in the periplasmic space may be to convert various C19 steroids to a common form required for binding and/or transport into the cell. Conversely, the inability of steroids to be bound and to support growth may reflect the absence of enzymes necessary for interconversion of these steroids to the form required for binding or transport. This mechanism would then necessitate only one, rather than multiple, transport system for various steroids. Such a model would also suggest great specificity for the steroid transport system.

We have recently reported a testosterone transport system in this organism using membrane vesicles prepared from induced bacteria [3]. The studies suggested specificity of the transport system, in that testosterone and dihydrotestosterone were transported, but not androstanedione or androstenedione (Watanabe, M. and Watanabe, H., unpublished observations). The intravesicular steroids were androstenedione and androstanedione when the steroids transported were testosterone or dihydrotestosterone, respectively. We have concluded that 17β-hydroxysteroid dehydrogenase activity was involved in the transport process. These observations raise the question of the functional role of enzymes and receptor proteins in the periplasmic space. Why do they not convert the various steroids to testosterone or dihydrotestosterone and accumulate these steroids in the periplasmic space? The possibility exists that under the conditions of growth this does in fact happen but that under conditions of our assay, the equilibrium constant of the 17β-hydroxysteroid dehydrogenase activity may be altered. This enzyme activity is reversible, requires NAD^+ for oxidation of the steroid and NADH for the reverse reduction reaction.

A second possible explanation for this apparent discrepancy is that the receptor protein is the membrane carrier protein released into the supernatant by osmotic shock and the bound steroid therefore represents the result of a "transport activity" *in vitro*. Testosterone may bind preferentially to such a carrier protein, but be subsequently released as androstenedione as happens under *in vivo* conditions. In this transport system, we envisage a high affinity for testosterone and dihydrotestosterone and a low affinity for androstenedione and androstanedione causing the release of the latter steroids into the intracellular compartment. If this mechanism were to operate *in vitro*, androstenedione and/or androstanedione would be expected to accumulate in the medium. Clarification of the nature and role of the "binding protein" will await its purifica-

tion and elucidation of the various components of the steroid transport process.

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