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TESTOSTERONE UPTAKE BY MEMBRANE VESICLES OF *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

Uptake of testosterone was demonstrated in membrane vesicles prepared from *Pseudomonas testosteroni* grown on testosterone. In contrast, membrane vesicles from uninduced cultures revealed no significant transport activity for steroids. The K_m of the reaction was $2 \cdot 10^{-6}$ M and the V 28.5 nmoles/min per mg protein. Steroid uptake was maximal within the pH range of 8 to 9 and at incubation temperatures between 30 and 37 °C. Transport of steroid was dependent upon NAD^+ and was reduced by NADH, dinitrophenol, and inhibitors of electron transport, such as $N_3^- \cdot CN^-$ and amytal. The intravesicular steroid concentration was approx. 800 times the steroid concentration present in the medium at the start of the incubation.

INTRODUCTION

Current investigations indicate that steroid hormones act by regulating the activity of certain genes in the cell nuclei of target tissues. After passing through the outer plasma membrane, the steroid is found in association with a specific receptor protein in the cytoplasm and the hormone–receptor complex is then transported into the cell nucleus where various steroid hormones have been found closely associated with the nuclear chromatin. Although the mode of steroid transport across the nuclear and plasma membranes can conceivably serve an important regulatory function in hormone action, few attempts have been made to examine the transport process in detail.

Bacterial systems have proven extremely useful in investigating the mechanism, the biochemistry and the genetics of transport systems for various sugars, amino acids and ions [1–3]. For the study of steroid transport across membrane barriers, we have utilized the organism *Pseudomonas testosteroni*, a bacterium capable of growth on various C19 and C21 steroids [4–6]. Induction of periplasmic steroid receptor proteins [7, 8], as well as steroid dehydrogenase activities [9], has been demonstrated during adaptive growth in this organism. In further pursuit of our investigations concerning the mechanism and regulation of steroid transport across cellular mem-

branes, we have isolated membrane vesicles from this organism. With this preparation, difficulties encountered with the intact organism due to extensive intracellular steroid metabolism [8], have been largely obviated. Such preparations have been used extensively to study bacterial transport mechanisms [10], but to date, steroid transport has not been examined. In this paper, we report the presence of an inducible transport system for testosterone in membrane vesicles prepared from cultures of *P. testosteroni*.

MATERIALS AND METHODS

Radioactive steroids, ^3H_2O, and carboxyl-^{14}C-labeled dextran (M_r 60 000–90 000) were obtained from New England Nuclear Corporation, Boston, Mass. Phenazine methosulfate was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisc., U.S.A.; NAD^+, NADH, NADP and NADPH from Schwarz–Mann, Orangeburg, N. Y.; unlabeled steroids from Steraloids, Inc., Pawling, N. Y.; and B-6 membrane filters (0.45 μm) from Schleicher and Schuell, Inc., Keene, N. H.

Preparation of membrane vesicles

P. testosteroni 11996, obtained from American Type Culture Collection, Rockville, Md., was grown at 30 °C in a medium containing per liter: 1 g $NH_4H_2PO_4$, 1 g $(NH_4)_2HPO_4$, 10 g Difco yeast extract and 10 ml of a solution containing per liter: 20 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 0.5 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 3H_2O$, 0.05 g $CuSO_4 \cdot 5H_2O$ and 10 ml 0.05 M H_2SO_4. When the cultures reached stationary phase growth ($A_{660\ nm}$ of 2.0), testosterone was added to a final concentration of 0.5 g per liter and the cultures were further incubated at 30 °C for 16 h ($A_{660\ nm}$ of 3.0, titre of 10^{10} bacterial cells/ml). The bacterial cells were harvested by centrifugation and membrane vesicles prepared after spheroplast formation using the lysozyme–EDTA method, as described by Kaback [11].

The number of surviving bacteria present in the membrane preparation was determined by plating. The titer of surviving bacteria in the final membrane preparation was 10^4 cells/ml. The transport of steroids by whole bacterial cells was examined in order to exclude the possibility that the measured steroid uptake in the transport studies reflected uptake by these surviving cells. Using concentrations of whole cells equivalent to or greater than the survivors in the membrane preparation, uptake of steroids could not be detected due to the efficient steroid-degradative activity of induced cells, which rapidly converted the tritiated steroid to ^3H_2O [8, 12]. These studies indicated that uptake by vesicle preparations was not due to the presence of whole cells.

Membrane-protein concentration was determined by the method of Lowry et al. [13].

Transport studies

A typical incubation mixture for transport studies contained in 0.2 ml: 50 μmoles of Tris–HCl, pH 9.0, approx. 170 pmoles of an aqueous solution of [7-^{3}H]-testosterone ($3.3 \cdot 10^5$ dpm/nmole), 200 nmoles of NAD^+ and 2 μg of membrane protein. The reaction mixture was incubated at 25 °C for 1 min, unless otherwise indicated, and the incubation was terminated by the addition of 5 ml of 0.5 M LiCl.

The reaction mixture was filtered immediately on Schleicher and Schuell membrane filters and washed once with 5 ml of 0.5 M LiCl. The filters were dried and the radioactivity was assayed in a liquid scintillation spectrometer.

Estimation of vesicle volume

To 4, 6 and 8 ml of membranes prepared from induced cells (1.0 mg of protein/ml), 50 μl of carboxyl-^{14}C-labeled dextran (10 μCi/ml) and 5 μl of $^{3}H_2O$ (100 μCi/ml) were added and the volume was made up to 9.0 ml with 50 mM Tris, pH 9.0. The reaction mixture was incubated at 25 °C for 5 min and the membranes were harvested by centrifugation at 45 000 $\times g$ for 30 min. The supernatant and the pellet were assayed for ^{3}H and ^{14}C radioactivity in a liquid scintillation spectrometer. The interstitial volume between the vesicles was determined by the amount of ^{14}C-labeled dextran present in the pellet, and the total water by the amount of $^{3}H_2O$ in the pellet. The total water minus the interstitial volume provided the internal vesicular volume.

RESULTS

Steroid uptake by membrane vesicles

Membrane vesicles were isolated from stationary-phase cultures of *P. testosteroni* grown either in the presence or absence of testosterone. There was a small amount of uptake of [^{3}H]testosterone by membrane vesicles prepared from cells grown in the absence of testosterone (Fig. 1). In membrane vesicles prepared from induced

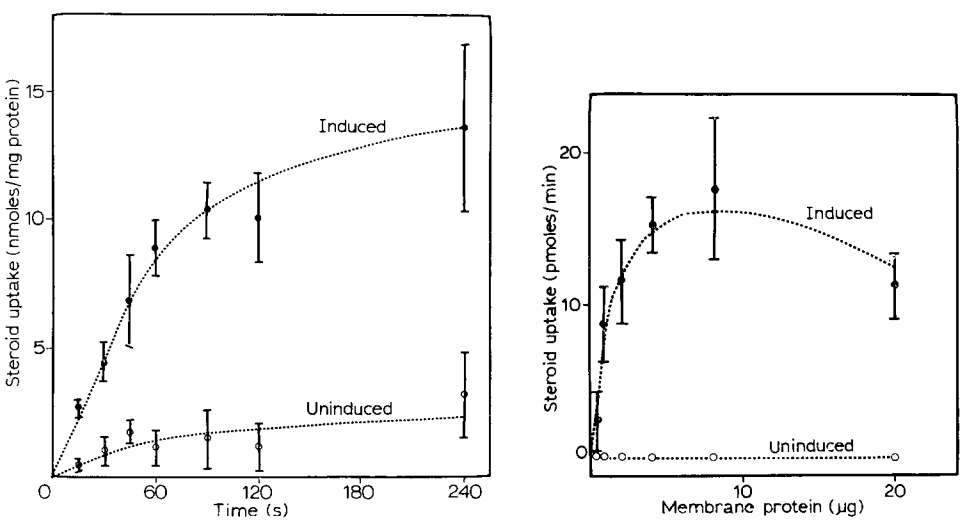

Fig. 1. Uptake of steroid by membrane preparations of induced and uninduced *P. testosteroni*. The reaction mixture contained in 0.2 ml, 50 μmoles of Tris–HCl, pH 9.0, 166 pmoles of [7-^{3}H]testosterone ($3.3 \cdot 10^5$ dpm/nmole), 200 nmoles of NAD^+, and 2 μg of membrane protein prepared from uninduced bacteria or bacteria induced for 16 h by growth on testosterone. The reaction mixture was incubated at 25 °C for periods up to 2 min. Each value shown represents the mean of five experiments $\pm$S.E.

Fig. 2. Uptake of steroid using various amounts of membrane preparation. The assay was performed as described in Fig. 1, except that various amounts of membrane protein were added and the incubation was terminated at 1 min. The values shown represent the mean of five experiments $\pm$S.E.

cells, there was an initial rapid uptake during the first minute of incubation, with a somewhat slower uptake thereafter. Examination of the initial phase demonstrated that uptake was dependent upon the amount of membrane protein present in the incubation mixture (Fig. 2).

Effect of NAD^+ on transport

The uptake of testosterone by membrane vesicles was variable and small in the absence of NAD^+ (Fig. 3). The addition of NAD^+ to induced membrane preparations resulted in a stimulation of steroid uptake, with maximal stimulation occurring at concentrations above 0.1 mM. No such stimulation was observed in membrane vesicles from uninduced cells.

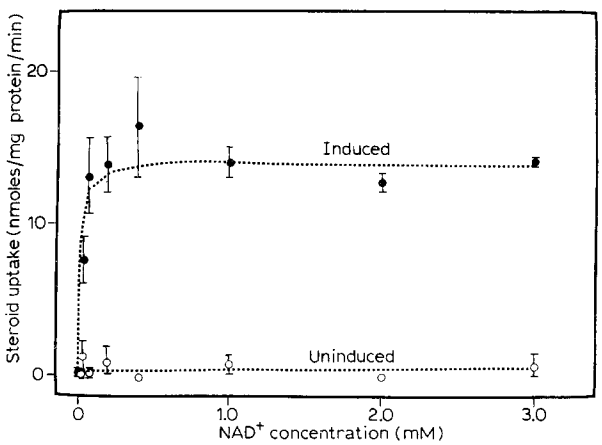

Fig. 3. Requirement for NAD^+ for steroid uptake by membrane preparations. The assay was performed as described in Fig. 1, except that the NAD^+ concentration varied from 0 to 3 mM and the incubation was terminated at 1 min. The results shown are the means of five experiments.

The effect of D-lactate, succinate, glucose, *P*-enolpyruvate, ATP, cyclic AMP, NADH, NADP, and NADPH was examined but none of these energy sources was found to stimulate steroid uptake (Table I). D-Lactate and NADH added in a concentration of 20 mM inhibited the stimulatory effect of 1 mM NAD^+, NADH producing the most marked inhibition.

Determination of rate constant

The initial rate of steroid uptake was examined at various external testosterone concentrations (Fig. 4). Over the range studied, a significant uptake was not demonstrable in membrane preparations from uninduced cells. In preparations from induced cells, the initial rate of steroid uptake increased rapidly with increase in the external concentration of testosterone up to approx. $1 \cdot 10^{-6}$ M. At concentrations above $1 \cdot 10^{-6}$ M there was a decline in the rate of increase. The process of steroid uptake in the presence of NAD^+ was characterized by a K_m of $2 \cdot 10^{-6}$ M and a V of 28.5 nmoles/min per mg protein (Fig. 5).

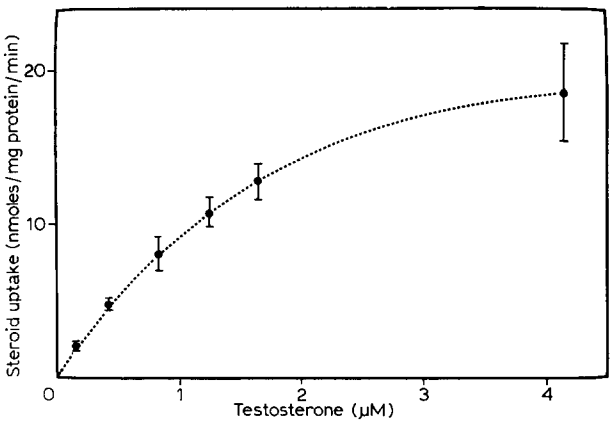

Fig. 4. Effect of external testosterone concentration on initial rate of steroid uptake by membrane preparations. The assay was performed as outlined in Fig. 1, except that the [7-^{3}H]testosterone concentration varied from 0.16 to 4.16 μM and the incubation was terminated after 1 min. The results shown are means obtained from five experiments.

TABLE I

EFFECT OF VARIOUS ENERGY SOURCES ON STEROID UPTAKE

The assay was performed as described in Fig. 1 except that the incubation was terminated after 1 min. Uptake was determined in the presence or absence of 1 mM NAD^+. The values shown are means derived from six experiments $\pm$S.E.

Additions	Concentrations (mM)	Steroid uptake (nmoles/min per mg protein)	
		Without NAD^+	With NAD^+
None		2.72±0.41	8.63±0.71
D-Lactate	20	2.23±0.64	5.45±1.09
Succinate	20	1.19±0.87	10.22±0.36
Glucose	20	1.09±0.39	8.19±1.22
P-Enolpyruvate	20	2.49±0.37	11.81±0.58
ATP	20	1.20±0.24	7.26±0.58
3′,5′-Cyclic AMP	8	0.42±0.23	6.74±0.59
NADH	20	2.34±0.75	1.55±0.64
NADP	5	2.77±0.48	6.32±0.89
NADPH	5	0.79±0.28	9.05±1.08

Requirements for steroid uptake

Steroid uptake was maximal between pH 8 and 9, with a dramatic fall below pH 8 (Fig. 6). The optimum temperature for uptake was between 30 and 37 °C (Fig. 7). The addition of EDTA or dithiothreitol to the reaction mixture did not influence steroid uptake. The effect of various ions was investigated, but no consistently significant stimulation or inhibition could be detected at the concentration tested (Table II).

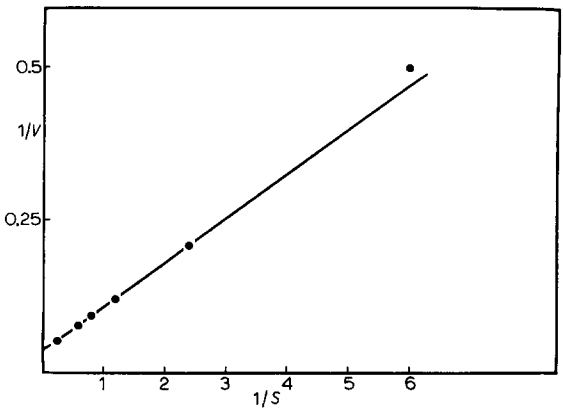

Fig. 5. Effect of external testosterone concentration on initial rate of steroid uptake by membrane preparations. The data presented in Fig. 4 are plotted according to the method of Lineweaver and Burk [23]. The value of $1/V$ represents the reciprocal of steroid uptake measured as nmoles/min per mg protein and the value of $1/[S]$ represents the reciprocal of testosterone concentration (μM).

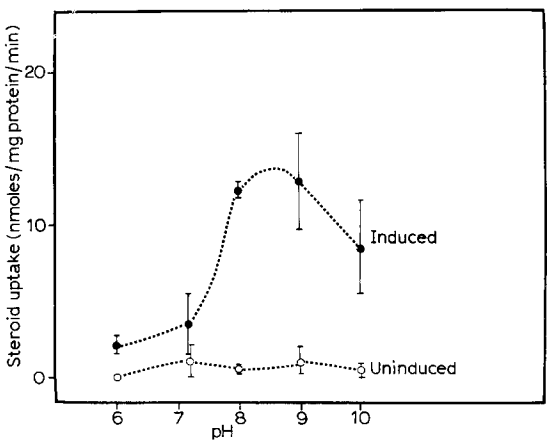

Fig. 6. Effect of pH on steroid uptake by membrane preparations. The assay was performed as described in Fig. 1, except that pH of the Tris buffer added to the assay mixture varied between 6 and 10 and the incubation was terminated after 1 min. The results shown are means obtained from three experiments.

Effect of inhibitors on steroid uptake by membrane vesicles

The effect of known inhibitors of the electron-transport chain was investigated. NaN_3, KCN and sodium amytal produced a pronounced inhibition (Table III). Dinitrophenol, an uncoupler of oxidative phosphorylation, also markedly inhibited testosterone transport. The sulfhydryl reagents, *N*-ethylmaleimide and *p*-chloromercuribenzoate, had no significant effect. Transport of amino acids by membrane vesicles has been reported to be light sensitive [14]. Steroid transport by membrane vesicles prepared from *P. testosteroni* was also inhibited by sunlight (Fig. 8).

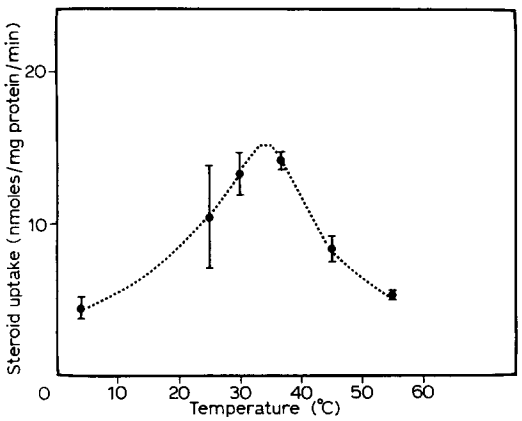

Fig. 7. Effect of incubation temperature on steroid uptake by membrane preparations. The assay was performed as described in Fig. 1, except that the incubation temperature was varied from 4 °C to 55 °C and the incubation was terminated after 1 min. The results shown are means obtained from three experiments.

TABLE II

EFFECT OF IONS, EDTA AND DITHIOTHREITOL ON STEROID UPTAKE BY MEMBRANE VESICLES

The assays were performed as described in Fig. 1 except that the incubation was terminated after 1 min. EDTA was added to a concentration of 25 mM and dithiothreitol and ions to a final concentration of 5 mM. The values shown are means derived from six determinations ±S.E.

Additions	Steroid uptake (nmoles/min per mg protein)		
	Expt 1	Expt 2	Expt 3
None	9.23±0.66	9.82±0.89	6.04±0.21
Dithiothreitol	9.33±0.89		
EDTA	8.63±0.71		
KCl		13.30±1.90	7.52±0.58
NaCl		8.78±1.37	5.98±0.55
$MgCl_2$		11.35±1.25	4.80±0.50
$MnCl_2$		8.45±0.62	5.51±0.10
$CoCl_2$		7.55±0.40	5.06±0.59
$FeCl_2$		8.54±0.79	4.33±0.48
$CaCl_2$		11.29±1.10	5.71±0.48
$MgSO_4$		11.60±0.97	6.20±0.41
$ZnSO_4$		6.14±0.43	4.39±1.04
NH_4HCO_3		10.41±1.36	6.78±0.29

Steroid concentration in membrane vesicles

The internal vesicle volume determined as described in Materials and Methods was 14.4 μl per mg of membrane protein which is higher than values reported for *Escherichia coli* and *Bacillus subtilis* [15, 16]. The intravesicular steroid concentration 1 min after incubation was approx. 800 times the steroid concentration present in the medium at the start of the incubation.

TABLE III

EFFECT OF INHIBITORS ON STEROID UPTAKE BY MEMBRANE VESICLES

The assay was performed as described in Fig. 1 except that the incubation was terminated after 1 min. The values shown are means derived from five experiments.

Addition	Concentration (M)	Steroid uptake (nmoles/min per mg protein)
None		20.55±2.45
KCN	$1 \cdot 10^{-2}$	6.62±1.48
NaN_3	$5 \cdot 10^{-3}$	19.67±2.54
	$1 \cdot 10^{-2}$	9.28±2.36
	$5 \cdot 10^{-2}$	4.84±0.18
Sodium amytal	$8 \cdot 10^{-3}$	5.04±1.81
	$4 \cdot 10^{-2}$	0
Dinitrophenol	$5 \cdot 10^{-4}$	5.05±0.54
	$1.2 \cdot 10^{-3}$	5.27±0.66
N-Ethylmaleimide	$2 \cdot 10^{-3}$	15.43±0.96
	$1 \cdot 10^{-2}$	16.19±1.86
p-Chloromercuribenzoate	$3.5 \cdot 10^{-4}$	17.39±2.82

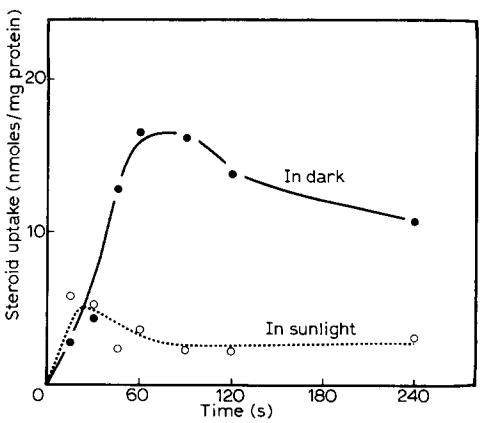

Fig. 8. Effect of sunlight on steroid uptake by membrane vesicles. The assay was performed as described in Fig. 1. The incubation was carried out in the presence or absence of sunlight for the time period indicated.

DISCUSSION

Since *P. testosteroni* is a bacterium capable of growth on various C19 and C21 steroids [4–6], we postulated that it should have an efficient transport system for such steroids. Although an inducible steroid-binding activity could be demonstrated during growth on testosterone [7, 8], steroid transport by whole bacterial cells was difficult to detect since induced enzymes degraded the steroid rapidly to CO_2 and H_2O [8, 12]. Since membrane vesicles prepared from various bacteria retain transport capabilities [10], testosterone transport was examined in membrane vesicles

prepared from *P. testosteroni*. Vesicles prepared from uninduced cultures did not concentrate testosterone, whereas preparations from cultures grown on testosterone for 16 h demonstrated uptake of steroid. Cultures induced for 16 h were used for this study since maximum induction of dehydrogenase activities occurred between 12 and 16 h [8]; it was felt that transport processes might also be maximally induced at this time. Cultures induced for 40 h were also tested since this time period coincided with the appearance of greatest levels of periplasmic steroid-binding activity. Although testosterone transport could be demonstrated with such membrane vesicles, the uptake was somewhat less than that observed with the 16-h preparation.

Since testosterone uptake was linear during the first minute of incubation, all studies were performed at 1 min, thus ensuring initial rate conditions. The K_m of the reaction was $2 \cdot 10^{-6}$ M and the V 28.5 nmoles/min per mg protein. These values are similar to those reported for transport of amino acids and sugars in other bacteria [10]. Testosterone uptake by membrane vesicles was saturable at steroid concentrations above 2–3 μM. The saturability of the system suggested that testosterone transport was a carrier-mediated process rather than passive diffusion.

The optimum temperature for testosterone uptake was between 30 and 37 °C. Although this organism is able to grow on other carbon sources at 37 °C, the optimum temperature for growth on testosterone is 30 °C [8]. The organism does not grow on testosterone at 45 °C [8]; this may be related to the inability to transport testosterone at this temperature. It is of interest that uptake by membrane vesicles was greater at 37 °C than at 30 °C. The optimum temperature of growth at 30 °C may be related to the integrity of the steroid–receptor protein complex in the periplasmic space. This complex is stable at 30 °C but reversibly inhibited at 37 °C [8].

The ratio of steroids inside and outside the vesicle was estimated. The intravesicular steroid at 60 s was 800 times that in the medium at the start of the incubation period. These results suggested an active transport process, leading to an accumulation of steroid against a concentration gradient. Further studies are in progress in order to determine the nature of the intravesicular steroids.

Transport of solutes appears to be linked to the electron-transport chain in many microorganisms [10, 15–19]. Transport is dependent upon an electron donor capable of entering the electron-transport chain, either directly (reduced phenazine methosulfate) or via a dehydrogenase (D-lactate, L-lactate, succinate or NADH), and is inhibited by inhibitors of the electron-transport chain. Transport of testosterone in *P. testosteroni* was also inhibited by N_3^-, CN^- and amytal and the mechanism of this inhibition remains to be elucidated. The physiological electron donor(s) for transport is not yet known in many cases, although membrane-bound α-glycerol-*P*-dehydrogenase, D-lactate dehydrogenase and NADH dehydrogenase have been implicated in various systems [10, 20]. The steroid transport system in *P. testosteroni* does not appear to be related to any of these dehydrogenase systems. Neither D-lactate nor NADH stimulated testosterone transport in membrane vesicles prepared from induced cells.

Transport of testosterone by membrane vesicles was dependent upon the electron acceptor NAD^+, and was inhibited by NADH. NAD^+ is known to be required in the following reversible reaction catalyzed by the induced enzyme, 17β-hydroxysteroid dehydrogenase [21]:

$$\text{Testosterone} + NAD^+ \rightleftarrows \text{Androstenedione} + NADH + H^+$$

It is of interest that the pH optimum for transport is between 8 to 9, which is also the pH optimum for the enzyme 17β-hydroxysteroid dehydrogenase [22]. Furthermore, membrane vesicles have been shown to contain this enzyme and NAD^+ is reduced with generation of androstenedione during or shortly after transport (Watanabe, M. and Watanabe, H., unpublished). These findings are suggestive of an integral role for 17β-hydroxysteroid dehydrogenase in the transport of testosterone. The exact role of this enzyme activity in testosterone transport remains to be elucidated. It is possible that the enzyme 17β-hydroxysteroid dehydrogenase is itself the transport protein for steroids. On the other hand, testosterone transport may be similar to sugar and amino acid transport systems except that, instead of D-lactate dehydrogenase, the steroid dehydrogenase is involved.

In summary, this is the first study demonstrating steroid transport across bacterial cell membranes. The transport process is inducible, is saturable and appears to be carrier-mediated, and appears to involve specific mechanisms similar to those reported for other transportable substances. Further studies are indicated in order to elucidate the mechanism and regulation of steroid transport across cell membrane. Membrane vesicles prepared from *P. testosteroni* offer an ideal system for such a study.

ACKNOWLEDGEMENTS

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