Journal of Steroid Biochemistry, 1976. Vol. 7, pp. 171–175. Pergamon Press. Printed in Great Britain

MEMBRANE BOUND 3β AND 17β-HYDROXYSTEROID DEHYDROGENASE AND ITS ROLE IN STEROID TRANSPORT IN MEMBRANE VESICLES OF *PSEUDOMONAS TESTOSTERONI*

MAMORU WATANABE* and LUISA PO

Division of Endocrinology and Metabolism, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

(*Received* 4 *August* 1975)

SUMMARY

Uptake of steroids by membrane vesicles prepared from *Pseudomonas testosteroni* was specific for testosterone, dihydrotestosterone and dehydroepiandrosterone. The intravesicular steroid was androstenedione when testosterone or dehydroepiandrosterone was transported and androstanedione in the case of dihydrotestosterone. During transport NAD^+ was reduced to NADH. These results indicated that steroid transport could best be described as a group translocation process and involved 3β and 17β-hydroxysteroid dehydrogenase activity. The steroid itself appeared to function as the physiological electron donor via NADH, generated during transport and oxidation of the steroid.

INTRODUCTION

Membrane vesicles prepared from induced cultures of *Pseudomonas testosteroni* have been shown to concentrate testosterone†, allowing detailed investigation of steroid transport across cell membranes. Testosterone uptake by membrane vesicles was saturable, suggesting that transport was a "carrier-mediated" process [1]. The intravesicular steroid concentration was 800 times the testosterone concentration in the medium at the start of the incubation suggesting an active transport system for accumulation of steroids. Transport of testosterone appeared to be dependent on energy generated by transfer of electrons, as judged by the inhibition of transport by cyanide and amytal.

Recent studies in *Escherichia coli* membrane vesicles indicate that transport of amino acids and sugars is coupled primarily to membrane-bound D-lactate dehydrogenase [2]. Steroid uptake in *P. testosteroni* was dependent upon NAD^+ and was optimal at a pH range between 8 and 9, similar to the requirements of 3β and 17β-hydroxysteroid dehydrogenase activity [3, 4]. In this study we have investigated the involvement of this membrane-bound enzyme activity in steroid transport.

EXPERIMENTAL PROCEDURES

Materials. P. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; unlabelled steroids from Steraloids, Pawling, New York; NAD^+ from Schwarz–Mann, Orangeburg, New York; radioactive steroids from New England Nuclear Corporation, Boston, Massachusetts. [7-^{3}H]-Androstenedione and [7-^{3}H]-1,4-androstadiendione were prepared by incubation of [7-^{3}H]-testosterone with membrane vesicles and purified and identified by thin-layer chromatography as described below.

Methods. Media used for growth and conditions for induction of binding activity have been described [5, 6]. Membrane vesicles were prepared after spheroplast formation using the lysozyme-EDTA method, as described by Kaback [1, 7]. Steroid transport was assayed as previously described [1]. Reaction mixtures for transport studies contained in 0·2 ml: 50 μmol of Tris–HCl, pH 9·0, 200 nmol of NAD^+, approx. 170 pmol of an aq. solution of labelled steroid, and 2 μg of membrane protein. The reaction mixture was incubated at 25°C for 1 min.

The assay for 3β and 17β-hydroxysteroid dehydrogenase was performed as described by Talalay [3]. Reduction of NAD^+ was determined by the change of absorbance at 340 nm.

Identification of transported steroids. Unless otherwise stated, the reaction mixture was identical to that used for transport studies except that the total vol. was 0·6 or 2 ml. At various times after incubation,

* Present address: Division of Medicine, University of Calgary, Calgary, Alberta.

† Trivial and systematic nomenclature of steroids: Androsterone—3α-hydroxy-5α-androstan-17-one; Aldosterone—11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al; Androstenedione—4-androstene-3,17-dione; 5α-Androstanedione—5α-androstane-3,17-dione; 1,4-Androstadiendione—1,4-androstadiene-3,17-dione; 5α-Dihydrotestosterone—17β-hydroxy-5α-androstan-3-one; Cortisol—11β,17,21-trihydroxy-4-pregnene-3,20-dione; Progesterone—4-pregnene-3,20-dione; Testosterone—17β-hydroxy-4-androsten-3-one; Dehydroepiandrosterone-3β-hydroxy-5-androsten-17-one; 17-Hydroxyprogesterone—17-hydroxy-4-pregnene-3,20-dione; Deoxycorticosterone—21-hydroxy-4-pregnene-3,20-dione.

5 ml of 0·5 M LiCl was added, and the incubation mixture was filtered and washed once with 5 ml of 0·5 M LiCl. The radioactivity remaining on the filter paper was washed 5 times with 5 ml of 50% ethanol which extracted all of the intravesicular steroid. The filtrate and the ethanol wash thus obtained were each extracted three times with equal volumes of redistilled ethyl acetate, which was then washed three times with distilled H_2O. The ethyl acetate extract was dried over anhydrous sodium sulphate, filtered through paper, and taken to dryness on a flash evaporator.

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 system benzene–absolute ethanol (19:1, v/v), hexane–ethyl acetate (75:25, v/v), ethyl acetate–cyclohexane (50:50, v/v), and alumina plates, using the system cyclohexane–ethyl acetate–acetic acid (6:4:0·01, by vol.). One cm. portions were scraped and assayed for radioactivity. Gas–liquid chromatography was carried out on extracts with a model 810F 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 per min; column temperature, 220°C. The effluent was assayed for radioactivity in a liquid scintillation spectrometer.

RESULTS

Uptake of various steroids by membrane vesicles. Uptake of testosterone by membrane vesicles prepared from induced cultures of *P. testosteroni* has been previously reported [1]. We have examined the ability of membrane vesicles to concentrate other C-19 and C-21 steroids. Concentrative uptake appeared to be specific for certain C-19 steroids. Testosterone, dihydrotestosterone and dehydroepiandrosterone were concentrated by membrane vesicles, whereas other C-19 steroids such as 4-androstenedione, 5α-androstanedione, androsterone and 1,4-androstadiendione were not transported (Table 1). Failure to detect uptake of androstenedione by membrane vesicles was not related to loss of radioactive label from the 1,2 position during transport since concentration of [7^3H]-androstenedione or [^{14}C]-androstenedione could not be demonstrated.

Table 1. Uptake of various C-19 steroids by membrane vesicles

Steroid	Steroid uptake
	nmoles/min per mg protein
[1,2-^{3}H]testosterone	30.9 ± 1.5
[1,2-^{3}H]dihydrotestosterone	15.7 ± 6.5
[1,2-^{3}H]androstenedione	0
[1,2-^{3}H]androstanedione	0
[1,2-^{3}H]androsterone	0
[7-^{3}H]androstadiendione	0
[7-^{3}H]dehydroepiandrosterone	7.9 ± 3.5

Note. The assay was performed as described in the text except that various radioactive steroids were used. The values shown are means derived from 3 experiments ± standard error.

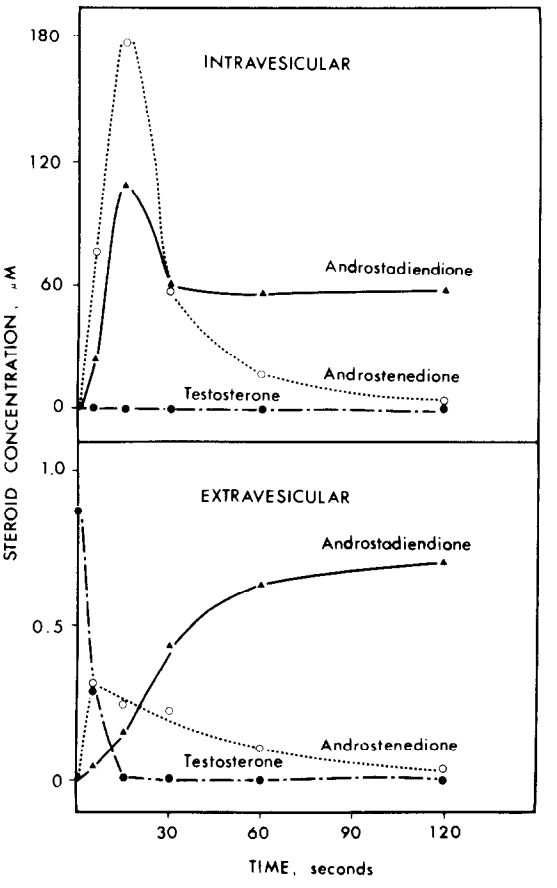

Fig. 1. Fate of [7-^{3}H]-testosterone during transport. The experiment was performed as described in Methods. The reaction mixture was identical to that of transport studies except that the concentration of membrane protein was 10 times that normally used and the total volume was 2·0 ml.

Steroids such as cortisol and aldosterone, which do not support growth of this organism [8, 9] were not concentrated by membrane vesicles. Thus far, we have been unable to demonstrate uptake of progesterone, 17α-hydroxyprogesterone or deoxycorticosterone, compounds which are known to support growth of this organism and to induce steroid degradative enzymes and steroid receptor proteins [6].

Characterization of the intravesicular steroid. To determine whether testosterone was metabolized during transport, steroids were extracted from the membrane vesicles and analyzed by thin-layer and gas–liquid chromatography. In order to obtain sufficient quantities of intravesicular steroids for identification, a membrane vesicle concentration 10-fold greater than that employed in normal transport studies was initially used. Under these conditions, testosterone disappeared rapidly from the medium, with approxi-

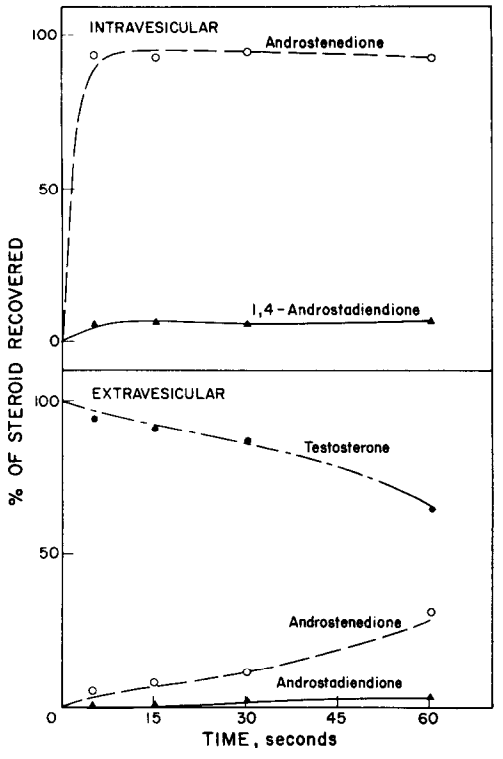

Fig. 2. Fate of [7-^{3}H]-testosterone during 60 s of incubation with greater testosterone concentration. The experiment was performed as described in Fig. 1 except that concentration of membrane protein was halved and steroid concentration was increased 5-fold in order to decrease the rate of conversion of the labelled testosterone during transport.

mately 1% remaining in the medium after 15 s (Fig. 1).

Testosterone could not be detected at any time in the membrane vesicle. Controls for non-specific binding of testosterone to membrane filters included assay mixtures without membrane vesicles, use of uninduced membrane vesicles, or reaction mixtures filtered immediately after addition of the various components. In multiple experiments, testosterone levels greater than in the control experiment were never observed. As testosterone disappeared from the external medium, 4-androstenedione and 1,4-androstadiendione appeared in the vesicle. Androstenedione reached higher intravesicular concentrations than androstadiendione during the first 30 s of incubation. With longer periods of incubation, androstenedione was almost totally converted to androstadiendione.

As testosterone disappeared in the external medium, 4-androstenedione appeared transiently, followed much later by increasing amounts of 1,4-androstadiendione. After 2 min, almost all of the steroid in the medium was androstadiendione.

The sequence of appearance of steroids during testosterone transport was also examined when testosterone disappeared much less rapidly from the external medium (Fig. 2). Again, testosterone could not be detected in the intravesicular compartment at any time. Androstenedione was the major steroid detected and accounted for more then 90% of the intravesicular steroid.

The intravesicular steroids obtained when dehydroepiandrosterone and dihydrotestosterone were the starting compounds were also examined. When dehydroepiandrosterone was added, the sequence of appearance of 4-androstenedione and 1,4-androstadiendione was identical to that observed with testosterone (Fig. 3). When dihydrotestosterone was added, however, 5α-androstanedione was the predominant intravesicular form (Fig. 4). Only small amounts of 1- and 4-androstenedione were detected, whereas 1,4-androstadiendione was not detectable in significant quantities at any time.

Membrane-bound 3β- and 17β-hydroxysteroid dehydrogenase and fate of NAD$^+$. Testosterone transport has been demonstrated to require NAD$^+$ [1]. Generation of NADH during testosterone uptake by membrane vesicles was demonstrated by measuring the spectral shift associated with reduction of NAD$^+$ (Fig. 5), which forms the basis for the measurement of 3β and 17β-hydroxysteroid dehydrogenase activity [3]. The NADH generation was linear with the amount of membrane protein added to the reaction mixture, and was dependent upon the presence of NAD$^+$ (Fig. 6). The maximum rate of NADH generation occurred at an NAD$^+$ concentration of 0·5 mM

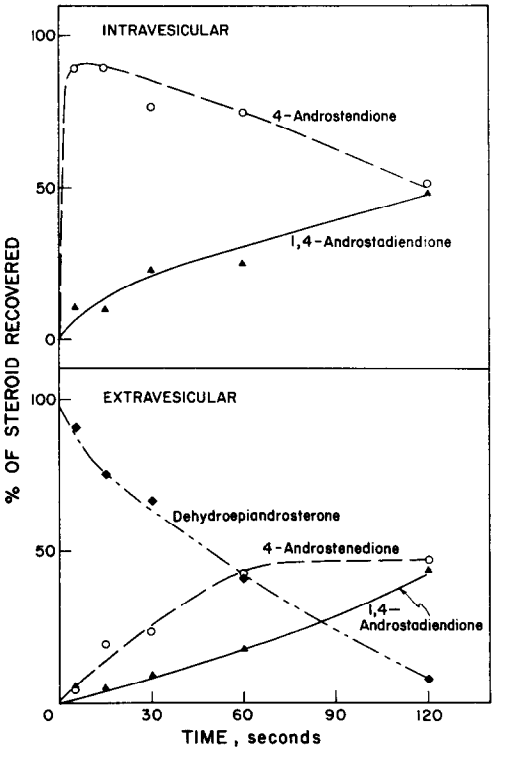

Fig. 3. Fate of [7-^{3}H]-dehydroepiandrosterone during transport. The intravesicular and extravesicular steroids during transport of dehydroepiandrosterone were analyzed as described in Methods. The reaction mixture was identical to that used for transport studies except that volume of reaction mixture was increased 3-fold.

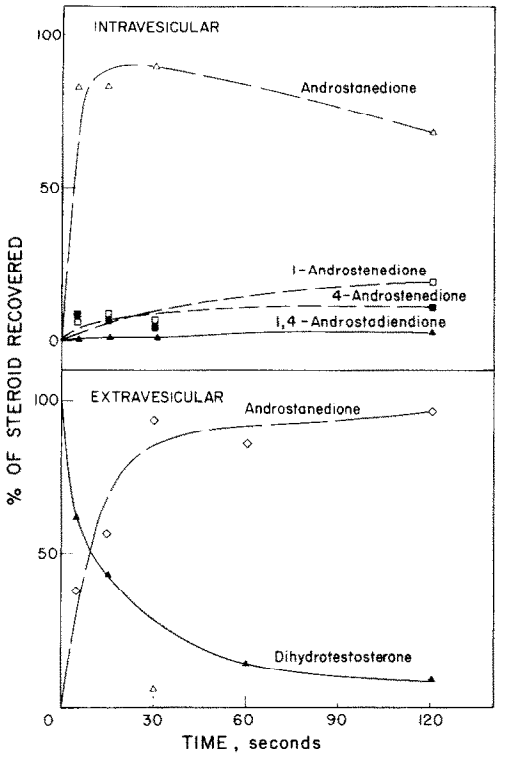

Fig. 4. Fate of [1,2-^{3}H]-dihydrotestosterone during transport. The intravesicular and extravesicular steroids during transport of dihydrotestosterone were analyzed as described in Methods. The reaction mixture was identical to that used for transport studies except that volume of reaction mixture was increased 3-fold.

or greater, which is similar to the concentration required for testosterone transport [1].

DISCUSSION

The demonstration of Kaback that membrane vesicles prepared from various bacteria retained transport capabilities have allowed detailed studies of membrane transport processes for various sugars, amino acids, and ions [2]. Transport of testosterone by membrane vesicles prepared from cultures of *P. testosteroni* [1] have allowed us to examine characteristics of steroid transport. Transport was specific for certain steroids. Testosterone, dehydroepiandrosterone and dihydrotestosterone were transported whereas compounds such as androstanedione, androstenedione and androsterone were not.

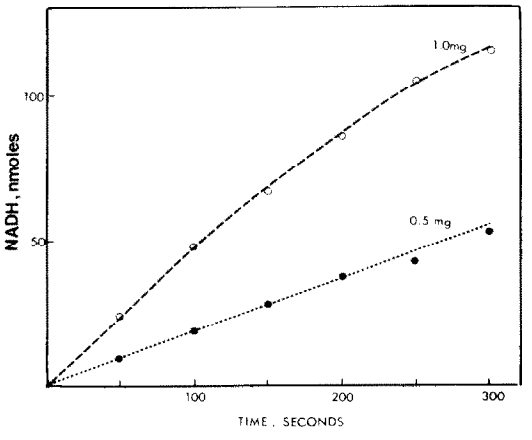

Fig. 5. Generation of NADH during testosterone transport. NADH was measured spectrophotemetrically as described by Talalay [3]. The reaction mixture contained in 1 ml: 250 μmol of Tris–HCl, pH 9·0, 40 nmol testosterone, 1 μmole of NAD^+, and 0·5 mg and 1·0 mg of membrane vesicle protein. The reaction mixture was incubated at 25°C and absorbance at A_{340} was determined in a spectrophotometer.

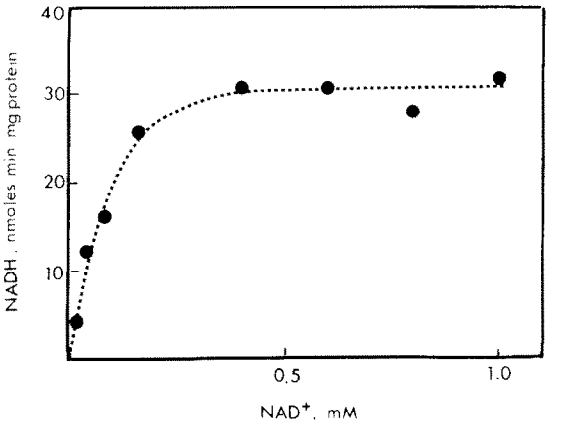

Fig. 6. Requirement for NAD^+ for NADH generation. The experiment was identical to that described in Fig. 5 except that 0·5 mg of membrane protein were added and NAD^+ concentration varied from 0–1 mM.

Testosterone did not appear to be transported as such. The intravesicular form was androstenedione, testosterone not being detectable at any time during the transport process. Similarly, when dehydroepiandrosterone was the starting steroid, the first intravesicular steroid detected was androstenedione. On the other hand, when dihydrotestosterone was the extravesicular steroid, the first and predominant intravesicular steroid detected was androstanedione. It was unlikely that these steroids were converted to androstenedione or androstanedione extravesicularly since these two steroids were not taken up by membrane vesicles. These studies, in addition to those demonstrating NADH generation during transport, indicated that 3β and 17β-hydroxysteroid dehydrogenase was closely linked to the transport of steroids. Steroid transport could be explained on the basis of group translocation, as in the case of the phosphotransferase system for sugar transport [10]. On the other hand, the results could also be explained by facilitative diffusion with steroid going down a concentration gradient. In this instance, the 3β and 17β-hydroxysteroid dehydrogenase activity would rapidly convert transported testosterone to androstenedione, keeping intracellular testosterone concentration at a low level and maintaining a downward concentration gradient at all times. The inability to detect even small amounts of testosterone intravesicularly leads us to favor the first of these two possibilities at this time.

Oxidation of D-lactate provides the primary driving force for the concentrative uptake of lactose and other

galactosides [11], amino acids [12], galactose [13], arabinose, glucuronate, gluconate, and glucose-6-phosphate [2], and in the presence of valinomycin, of potassium or rubidium [14]. Transport of steroids in *P. testosteroni* is also dependent on energy generated by transfer of electrons as judged by inhibition of transport by cyanide and amytal, but it is not related to oxidation of D-lactate [1]. The physiological electron donor for steroid transport may be NADH, generated from NAD^+ as testosterone is oxidized to androstenedione by the 3β and 17β-hydroxysteroid dehydrogenase activity.

Membrane vesicles prepared from induced cultures appear to have associated 1-ene-dehydrogenase activity. Androstenedione generated during transport of testosterone or dehydroepiandrosterone is transformed to androstadiendione. Androstanedione generated during dihydrotestosterone transport was converted to a small degree to 1-androstenedione. The limited and late conversion of steroids by this enzyme indicated that 1-ene-dehydrogenase activity was probably not essential for steroid transport.

Although *P. testosteroni* is able to grow on various C-19 and C-21 steroids, membrane vesicles prepared from induced organisms are able to transport only certain steroids such as testosterone, dihydrotestosterone or dehydroepiandrosterone. Periplasmic enzymes [15] may be necessary to transform the other steroids to the form in which they can be transported.

In summary, steroid transport in *P. testosteroni* demonstrates many of the characteristics reported for transport of sugars and amino acids [4] but with interesting differences. It is dependent upon 3β and 17β-hydroxysteroid dehydrogenase activity and it would appear that the steroid itself functions as the physiological electron donor *via* NADH production. At the present time we feel that the steroid transport can best be described as a group translocation process.

Acknowledgements—This investigation was supported by grant MT 4425 from the Medical Research Council of Canada, Ottawa, Canada. Dr. M. Watanabe is an Associate, Medical Research Council, Ottawa, Canada. The technical assistance of Mr. Ben Card is gratefully acknowledged.

REFERENCES

1. Watanabe M. and Po L.: *Biochem. biophys. Acta* **345** (1974) 419–429.
2. Kaback H. R.: *Biochim. biophys. Acta* **265** (1972) 367–416.
3. Talalay P.: In *Methods in Enzymology* (Edited by S. I. Colowick and N. O. Kaplan). Academic Press, New York. Vol. 5 (1962) 512–516.
4. Talalay P. and Dobson M. M.: *J. biol. Chem.* **205** (1953) 823–837.
5. Watanabe M., Phillips K. and Chen. T.: *J. steroid Biochem.* **4** (1973) 613–621.
6. Watanabe M., Phillips K. and Watanabe H.: *J. steroid Biochem.* **4** (1973) 623–632.
7. Kaback H. R.: In *Methods in Enzymology* (Edited by W. B. Jakoby). Academic Press, New York, Vol. 22 (1971) 99–120.
8. Talalay P., Dobson M. M. and Tapley D. F.: *Nature, Lond.* **170** (1952) 620–621.
9. Levy H. R., Talalay P.: *J. biol. Chem.* **234** (1959) 2009–2013.
10. Kundig W., Ghosh S. and Roseman S.: *Proc. natn. Acad. Sci., U.S.A.* **52** (1964) 1067–1074.
11. Barnes E. M. Jr. and Kaback H. R.: *Proc. natn. Acad. Sci., U.S.A.* **66** (1970) 1190–1198.
12. Kaback H. R. and Milner L. S.: *Proc. natn. Acad. Sci., U.S.A.* **66** (1970) 1008–1015.
13. Kerwar G. K., Gordon A. S. and Kaback H. R.: *J. biol. Chem.* **247** (1972) 291–297.
14. Bhattacharyya P., Epstein W. and Silver S.: *Proc. natn. Acad. Sci., U.S.A.* **68** (1971) 1488–1492.
15. Watanabe M. and Watanabe H.: *J. steroid Biochem.* **5** (1974) 439–446.