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3α-Hydroxysteroid Oxidoreductase Activities in Dihydrotestosterone Degradation and Back-formation in Rat Prostate and Epididymis

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The metabolism of dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-Adiol) was assessed in full homogenates of rat prostate and epididymis. The major degradational route of DHT was catalysed by the enzyme(s) 3α-hydroxysteroid oxidoreductase (HSOR). Enzyme kinetic characteristics V_{max}, K_m and V_{max}/K_m ratio, were obtained for the NADP(H)- and NAD(H)-dependent interconversion of DHT and 3α-Adiol at pH 7.0 and at saturated co-factor concentration. For both the reduction of DHT and the oxidation of 3α-Adiol, NAD(H) was the preferred co-factor when activities were rated by their V_{max} and V_{max}/K_m ratio. Combining the data with the earlier established V_{max}/K_m ratios for the 5α-reductase isozyme type I and II activities in rat prostate and epididymis indicated that DHT, at saturated co-factor concentrations, would not be sustained in either tissue considering the reported enzyme characteristics. The reported exclusive bioavailability of the co-factors NADPH and NAD^+ *in vivo*, however, will direct the metabolic pathways in these tissues to sustain the formation of DHT.

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INTRODUCTION

Growth and development of the rat prostate are androgen-dependent, dihydrotestosterone (DHT) being the most active androgen. Inhibition of the formation of DHT in the rat leads to apoptosis and cell death of prostatic cells [1]. This steroid is derived from testosterone (T) through 5α-reduction by the NADPH-dependent, membrane-bound 5α-reductase (5α-RED, E.C. 1.3.99.5) of which two isozymes exist in both rat and human [2]. The rat and human isozymes share similar pH profiles and affinity constants for several substrates [3, 4]. Earlier, our laboratory reported the quantification of the type I and type II 5α-RED by enzyme activity studies in rat prostate and epididymis [5].

DHT is metabolized by several enzymes [6, 7], the predominant DHT degrading enzyme in both rat prostate and epididymis being cytoplasmatic [8, 9] and/or nuclear [10] 3α-hydroxysteroid oxidoreductase (3α-HSOR, E.C. 1.1.1.50). In most male accessory 3sex tissues, 3β-HSOR (E.C. 1.1.1.51) only accounts for minor DHT metabolism [7, 11, 12]. 3α-HSOR is capable both of reducing DHT ($HSOR_{red}$) to 5α-androstane-3α,17β-diol (3α-Adiol) and back-oxidizing 3α-Adiol to DHT ($HSOR_{ox}$). Rat prostatic [13] and epididymal [14] HSOR enzymes are considered optimally active at neutral pH, like both 5α-RED subtypes [15].

In the present paper, the NADP(H)- and NAD(H)-dependent HSOR activities were quantified at neutral pH in rat prostate and epididymis tissue homogenates, both for the reduction of DHT to 3α/β-Adiol and the back-oxidation of 3α-Adiol to DHT, to get more insight into the role of these enzymes in the degradation and back-formation of the active androgen DHT.

MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]Testosterone (3.74 TBq/mmol) and [1α,2α(n)-^{3}H]17β-hydroxy-5α-androstan-3-one (dihydrotestosterone, DHT) (2.00 TBq/mmol) were purchased from Amersham (Amersham, U.K.). [9, 11-

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3H]5α-androstane-3α,17β-diol (Adiol) (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high performance liquid chromatography (HPLC) before use. Testosterone was purchased from Steraloids (Wilton, NH). Diethylether (p.a.), n-hexane (LiChrosolv) and 2-propanol (LiChrosolv) from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

Tissue preparation

Prostate and epididymis tissues were obtained from Wistar rats 7–13 weeks old and processed as described earlier [5, 16, 17]. In short, tissues were homogenized in a 20 mM phosphate buffer containing 1 mM monothioglycerol and 0.25 M sucrose using a Dounce tissue grinder. The homogenate was filtered through nylon netting of 50 and 140 mesh, respectively, to remove cell debris. The resulting full homogenate was used in all further experiments.

Enzyme assays and HPLC

The HSOR enzyme assays and subsequent separation of metabolites on HPLC were modified from the assay for 5α-RED described earlier [5, 16, 17]. In short, tritiated substrate steroids (either ^{3}H-DHT for $HSOR_{red}$ or ^{3}H-3α-Adiol for $HSOR_{ox}$) were isotopically diluted to the desired concentration with unlabelled steroid and incubated at 37°C in a 200 mM Tris-citrate buffer pH 7.0 with either 2 mM NADPH or NADH for $HSOR_{red}$, or with either $NADP^+$ or NAD^+ for $HSOR_{ox}$, in a final volume of 1 ml. The metabolites formed were extracted with diethylether and dissolved in 100 μl hexane for separation on HPLC. A Hibar LiChrosorb Diol column was used with an isocratic flow of 1.5 ml/min of hexane-propanol 96:4 (v/v). Radioactivity was monitored using a FloOne Beta Radiomatic A500 radio-chromatography detector. The percentile formation of either DHT or 3α/β-Adiol was measured to estimate initial velocities.

Calculation of enzyme characteristics

Initial velocities were estimated against a substrate concentration range of 50 nM to 3.2 μM, and K_m and V_{max} were calculated by computerized fitting (Enzfitter program) of the data using a non-linear regression procedure based on the Michaelis–Menten equation. Eadie–Scatchard plots of obtained initial velocity estimates were applied [5, 17]. The V_{max}/K_m ratio, used in this paper, can be considered a measure of enzyme activity at low (physiological) substrate concentrations, as at $[S] \ll K_m$, the Michaelis–Menten equation can be simplified to $v = V_{max}/K_m * [S]$.

RESULTS

Maximum velocity (V_{max}) and apparent affinity constant (K_m) values for HSOR activities were established from Eadie–Scatchard plots of the obtained estimated initial velocities in rat prostate and epididymis homogenates (Fig. 1). These values were obtained for the reduction of DHT to 3α/β-Adiol ($HSOR_{red}$), as well as for the back conversion of 3α-Adiol to DHT ($HSOR_{ox}$). Results were obtained at pH 7.0 for both NAD(H)- and NADP(H)-dependent metabolism at saturated (2 mM) co-factor concentrations.

For each of the NADP(H)- or the NAD(H)-dependent activities, the V_{max} values were approximately similar for both the reductive and oxidative pathways of HSOR in rat prostate as well as in epididymis (Table 1). The V_{max} values for NADH-dependent $HSOR_{red}$ were approximately 10-fold higher than the NADPH-dependent reductions in both tissues. The V_{max} for NAD^+-dependent $HSOR_{ox}$ was also more than 10-fold higher than the V_{max} for $NADP^+$-dependent oxidation in the rat prostate. In the rat epididymis, however, the V_{max} ratio of $NADP^+$- and NAD^+-dependent oxidation was only a factor 3. The affinity constants for the NADPH- and NADH-dependent $HSOR_{red}$ activities in both tissues were about 0.5-0.7 μM. The K_ms for $HSOR_{ox}$ were higher (approx. 0.8-7.3 μM).

The efficiency ratios V_{max}/K_m were established from the ordinate intercepts of the Eadie–Scatchard plots (Table 2). In both rat tissue homogenates the NAD(H)-dependent HSOR efficiency ratios were many times (7–18) higher than the NADP(H)-dependent activities at low substrate concentrations. In an earlier paper the V_{max}/K_m ratios for 5α-RED isozyme type I and type II activities in rat prostate and epididymis were described [5]. Total 5α-RED (type I and type II) V_{max}/K_m values are presented in Table 2 for purposes of comparison. 5α-RED activity is completely dependent on NADPH as co-factor.

Of the enzyme activities we have measured in the rat prostate and epididymis, both 5α-RED isozymes and $HSOR_{ox}$ contribute to the formation of DHT, whereas $HSOR_{red}$ degrades DHT to Adiol. Comparison of the V_{max}/K_m values of 5α-RED and $HSOR_{ox}$ with $HSOR_{red}$ in Table 2 approximates relative DHT-forming and degrading activities at low substrate concentrations. Total $HSOR_{red}$-activity (NADPH- and NADH-dependent) exceeds 5α-RED activity by more than 80-fold in rat prostate. In rat epididymis, the total 5α-RED activity found was much higher than in the prostate and total $HSOR_{red}$ potential *in vivo* activity exceeded total 5α-RED activity only approximately by a factor of 2 in this tissue. The ratio of the metabolism of DHT to Adiol ($HSOR_{red}$) over the back-conversion from 3α-Adiol to DHT ($HSOR_{ox}$) was approximately 2.4 and 5.9 in rat prostate and epididymis, respectively. Total potential *in vivo* DHT-degrading activity (V_{max}/K_m of $HSOR_{red}$) was 2354 and 608 μl/(min*mg protein) in rat prostate and epididymis, respectively, whereas the total V_{max}/K_m values of DHT-forming activities (total 5α-RED and $HSOR_{ox}$) were 996 and 366 μl/(min*mg protein), respectively (Fig. 2A and

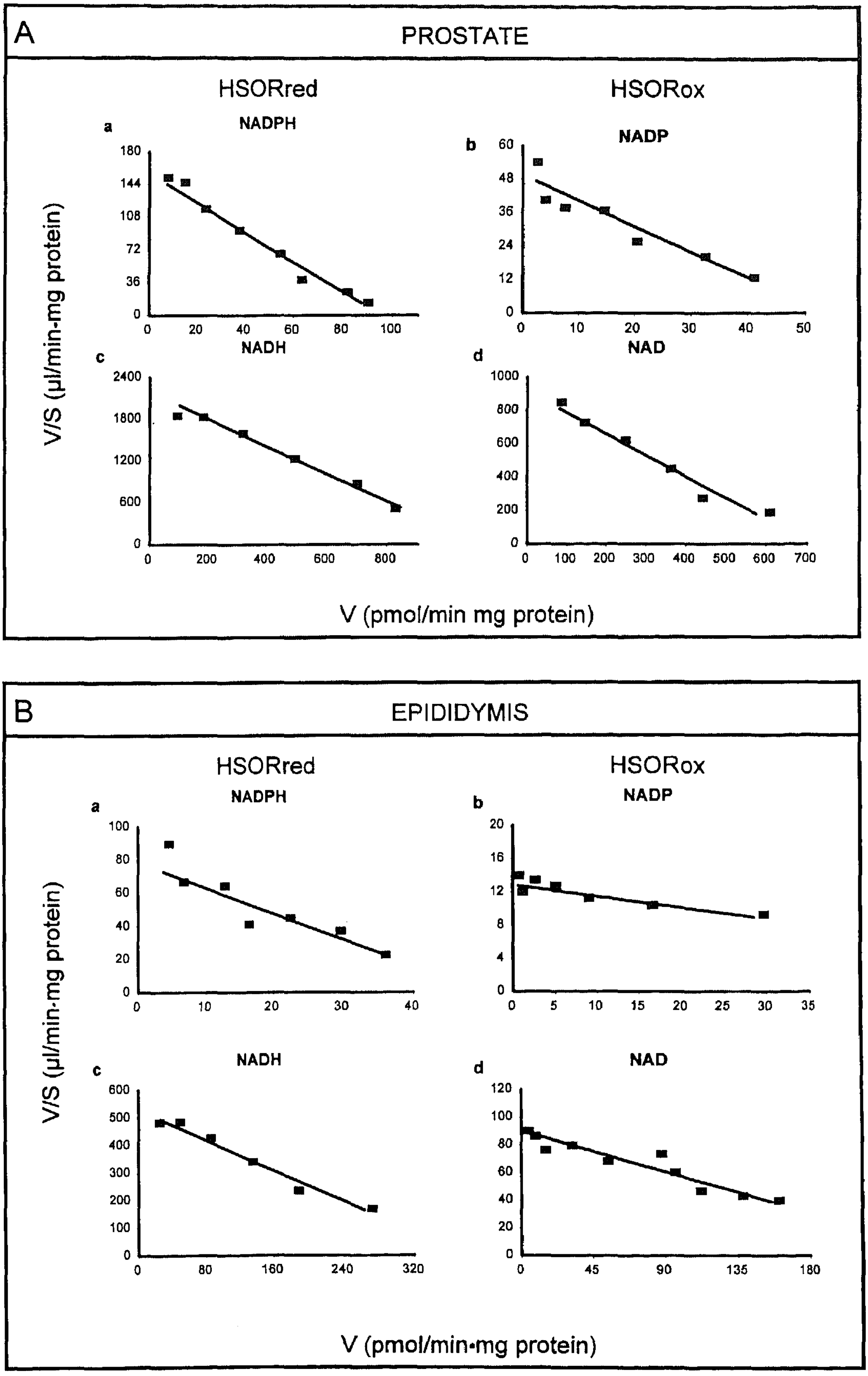

Fig. 1.Eadie–Scatchard plots of HSOR activities obtained in (A) rat prostate and (B) rat epididymis homogenates. Initial velocities were estimated for the reduction of DHT to Adiol ($HSOR_{red}$) (subfigures a and c) and the oxidative back formation of DHT from 3α-Adiol ($HSOR_{ox}$) (subfigures b and d). Both the NADP(H)- (subfigures a and b) and NAD(H)-dependent (subfigures c and d) activities were assessed. In this plot, the abscissa intercept denotes the maximum velocity, V_{max}, whereas the ordinate intercept denotes potential *in vivo* activity, V_{max}/K_m. The slope in this plot equals $-1/K_m$.

Table 1. V_{max} and K_m values for $HSOR_{red}$ and $HSOR_{ox}$ in rat prostate and epididymis

Tissue	Co-factor	$HSOR_{red}$		$HSOR_{ox}$	
		V_{max} pmol/(min*mg protein)	K_m nM	V_{max} pmol/(min*mg protein)	K_m nM
Prostate	NADP(H)	96	614	54	1090
	NAD(H)	1110	505	716	781
Epididymis	NADP(H)	51	645	94	7331
	NAD(H)	384	726	275	3042

Both the NADP(H)- and NAD(H)-dependent activity kinetic characteristics were determined in homogenates at pH 7.0 as also shown in Fig. 1.

2C). So, at pH 7.0, the potential *in vivo* activity, as measured by the V_{max}/K_m ratio, of degradation of DHT to Adiol predominated over the formation of DHT from T and the oxidative back-conversion from 3α-Adiol at saturated (2 mM) co-factor concentrations in both tissue homogenates (Fig. 2). However, when only the NADPH- and NAD^+-dependent activities were compared, considering the exclusive bioavailablity of these co-factors *in vivo* (*vide infra*), DHT accumulation would be sustained in both tissues (Fig. 2B and D).

DISCUSSION

In this study the reduction of DHT to Adiol and the back-oxidation of 3α-Adiol to DHT was investigated in rat prostate and epididymis homogenates. These steps in testosterone metabolism are performed by multiple isozymes of 3α- and 3β-hydroxysteroid-oxidoreductase (HSOR). There are ample reports about the presence and co-factor dependency of these enzymes in both rat prostate [7, 10, 12, 18, 19] and epididymis [9, 14, 20, 21].

In our hands, formation of 3β-Adiol was not detected, but the possibility could not be unequivocally excluded that 3α- and 3β-Adiol epimeres were not separated with the applied HPLC system. However, in all experiments only a single Adiol peak was detected which eluted with the same retention time as tritiated 3α-Adiol. When the mobile phase was changed to hexane/propanol 98:2 v/v, thereby more than doubling the retention times, still no additional peaks could be detected. Furthermore, the affinity constants found in the rat prostate and epididymis homogenate for $HSOR_{red}$ (approx. 0.5-0.7 μM) and $HSOR_{ox}$ (0.8-7.3 μM) were comparable to those found in literature for 3α-HSOR activity [8, 10, 18, 19]. The affinity constant reported for rat prostatic 3β-HSOR activity, 25.4 μM [18], is quite different from the K_ms of $HSOR_{red}$ activity ascertained in the present study, indicating that most probably only 3α-HSOR activity was measured. According to the literature, in most male accessory sex tissues, including the rat prostate, 3β-HSOR only accounts for minor DHT metabolism [7, 11, 12]. 3β-$HSOR_{red}$ activity is reportedly mainly located in the dorsal and lateral prostate and is not detectable in the ventral lobe [7, 12, 18]. In total rat prostate homogenate 3β-HSOR activity might thus have been under the detection limit of our assay. Finally, as the Eadie–Scatchard plots of HSOR activities were basically linear, the conclusion seems to be justified that we have measured a single enzyme activity, most probably 3α-HSOR. The $HSOR_{ox}$ activities were measured with tritiated 3α-Adiol as substrate and are therefore attributable only to 3α-$HSOR_{ox}$.

In the present study, an approximately 10-fold higher V_{max} was ascertained for the NAD(H)-dependent activities of both $HSOR_{red}$ and $HSOR_{ox}$ than for the NADP(H)-dependent activities. The NAD^+-dependent $HSOR_{ox}$ activity in the rat epididymis, however, was only three times the value of the $NADP^+$-dependent $HSOR_{ox}$. These data are seemingly at variance with some earlier reports that indicated NADP(H)-dependent HSOR activity to be greater than the NAD(H) activity in rat prostatic cytosol [8, 13]. However, when only the ventral prostate was assessed (800 × *g* supernatant), the NAD(H)-dependent HSOR activity was found to exceed the NADP(H)-dependent activity [7, 12]. Furthermore, in the rat prostate a nuclear-bound NADH-dependent $HSOR_{red}$ activity has been reported, being 20-fold higher than NADPH-dependent activity [10]. Two distinct 3α-HSOR activities have been found in the rat pituitary, one cytosolic preferring NADPH, another membrane-bound with NADH as co-factor [22, 23]. As in the present study a full homogenate was used, differences between our study and those in the literature might be

*Table 2. V_{max}/K_m ratios (μl/(min*mg protein)) for rat prostate and epididymis homogenates of the enzymes 3α-hydroxysteroid-oxidoreductase, the reductive ($HSOR_{red}$) and oxidative ($HSOR_{ox}$) pathway, and 5α-reductase type I and II (5α-RED) [5]*

Tissue	Co-factor	$HSOR_{red}$	$HSOR_{ox}$	5α-RED
Prostate	NADP(H)	156	50	29
	NAD(H)	2198	917	—
Epididymis	NADP(H)	79	13	263
	NAD(H)	529	90	—

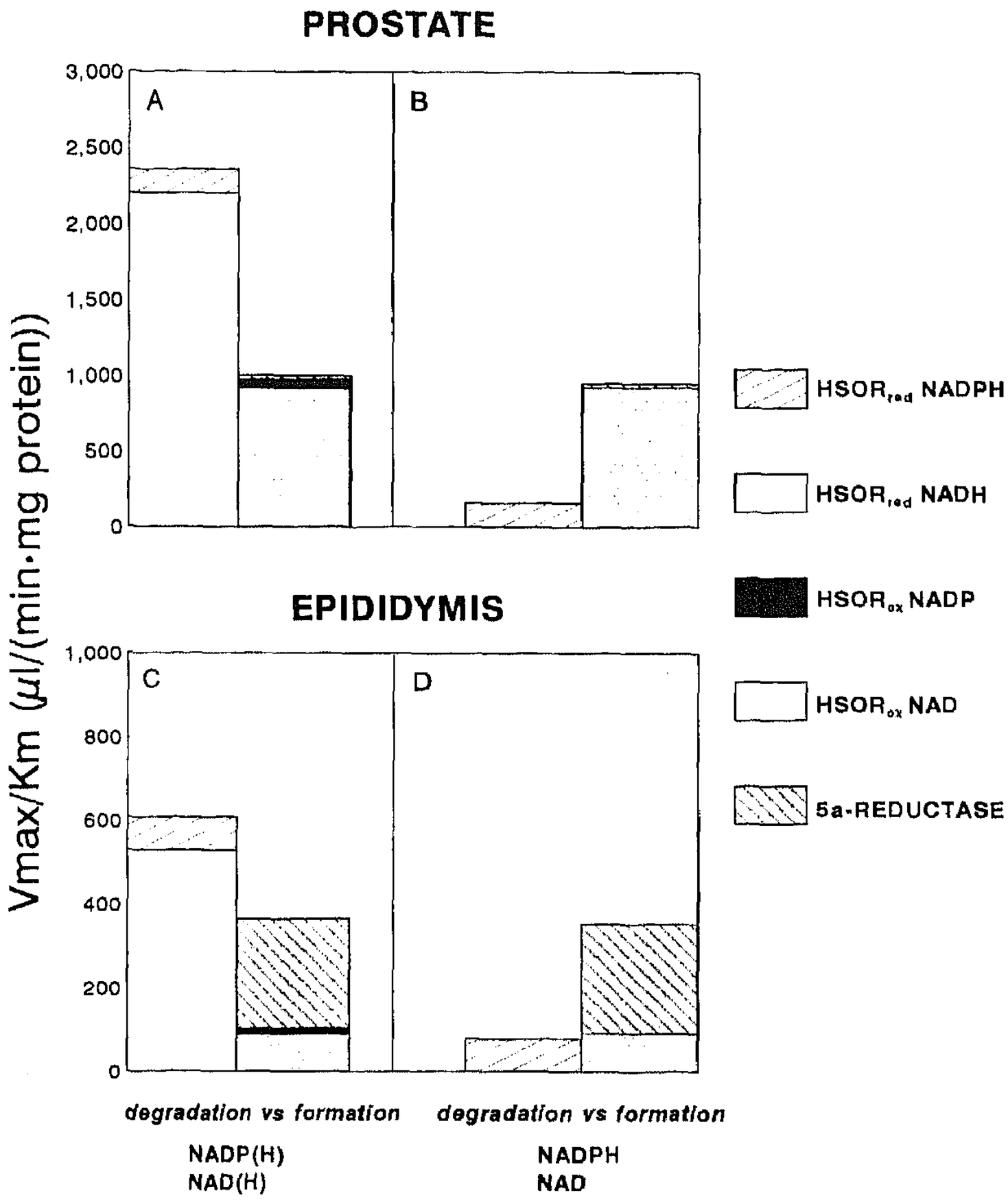

Fig. 2. Potential *in vivo* activities (V_{max}/K_m in $\mu l/(min*mg\ protein)$) obtained in rat prostate (A and B) and in rat epididymis (C and D) homogenates. Left bars in each subfigure denote DHT-degrading enzyme activities ($HSOR_{red}$), right bars denote DHT-forming activities (total 5α-RED and $HSOR_{ox}$). A and C: All enzyme activities (NADP(H)- and NAD(H)-dependent) reported in this paper obtained as described in the Materials and Methods section. B and D: NADPH- and NAD^+-dependent enzyme activities, thus DHT metabolism at "*in vivo*" cellular cofactor concentrations: NADPH ⩾ $NADP^+$ and NAD^+ ⩾ NADH.

due to the experimental protocol applied: unlike in our study, in many others cytosolic fractions were used or homogenates were centrifuged at $800 \times g$, leading to the loss of any nuclear- or membrane-bound NADH-dependent HSOR activity.

$HSOR_{ox}$ activity has been reported to exceed that of $HSOR_{red}$ both for their maximum velocities in the ventral prostate [7], as for enzyme activities in cell cultures at low substrate concentrations (50 nM) [6]. Unlike in the former study, the V_{max} values for both $HSOR_{red}$ and $HSOR_{ox}$ activities reported in the present paper for whole prostate homogenates were approximately similar, which is more in line with recent data reported for the ventral prostate [12]. However, due to the lower affinity for the back conversion of 3α-Adiol to DHT, the total enzyme efficiency ratios (V_{max}/K_m) reported here favoured degradation of DHT at low (i.e. physiological) substrate concentrations. The activities at these low substrate concentrations, as reported by Orlowski and Clark [6], should be comparable with our V_{max}/K_m ratios, as this ratio indicates enzyme activity at $[S] \ll K_m$ [24]. However, in the study of Orlowski and Clark a cell culture was used where no co-factor was added, whereas our results were obtained at saturating co-factor concentrations. The difference between our study and theirs might in fact be attributable to the limited bioavailability of co-factor in mammalian cells [25, 26] (*vide infra*).

The results presented in this paper do not favour the accumulation of DHT in rat prostate. The high $HSOR_{red}$ V_{max}/K_m ratios indicated a higher tissular 3α-Adiol concentration under steady-state conditions (Fig. 2A). The V_{max}/K_m ratios for $HSOR_{red}$, $HSOR_{ox}$ and total 5α-RED obtained in the rat epididymis also do not favour the accumulation of DHT (Fig. 2C). Reportedly, however, tissular co-factor concentrations *in vivo* are NADPH ⩾ $NADP^+$ and NAD^+ ⩾ NADH [25, 26]. These relative concentrations would therefore favour the reduction of DHT by the NADPH-dependent $HSOR_{red}$ and the back oxidation by the NAD^+-depe-dent $HSOR_{ox}$. This profoundly changes the interpretation of our results (Fig. 2). As the NAD(H)-dependent HSOR activities were much higher than the NADP(H)-dependent activities, the *in*

vivo cellular co-factor concentrations would favour DHT formation, in sharp contrast to results obtained at saturated co-factor concentrations *in vitro*. Early reports about the *in vivo* injection of tritiated androgens indeed showed an accumulation of ^{3}H-DHT in the rat prostate [27–29], but an accumulation of tritiated 3α-Adiol in the epididymis [29]. The latter observation might indicate that the tissular co-factor concentrations in rat epididymis are different from those in the prostate and from those generally assumed *in vivo*. These results indicate that a small amount of NADH will lead to extensive degradation of DHT, because of the high NADH-dependent $HSOR_{red}$ capacity of these tissues. Thus, NAD(H) may serve as a potent regulatory factor in the degradation and back formation of DHT in these rat tissues, as has been proposed for NADPH in the human (hyperplastic) prostate [30].

In summary, in this paper reductive and oxidative 3α-HSOR activities have been described in rat prostate and epididymis homogenates. Both the NADP(H)- and NAD(H)-dependent HSOR activities were assessed. Comparison of the enzyme activities involved in formation and degradation of DHT in these tissues, indicated that DHT concentration would not be sustained in either tissue at saturated co-factor concentrations. Exclusive bioavailability of the co-factors NADPH and NAD^+ in intact cells *in vivo*, however, would direct the metabolic pathways in these tissues to the formation of DHT.

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