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THE TRANSFORMATION OF TESTOSTERONE BY *TETRAHYMENA PYRIFORMIS*

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SUMMARY

[4-^{14}C]-Testosterone was completely transformed into multiple ^{14}C-products when incubated with cultures of *Tetrahymena pyriformis*, strains W and II-1. One of the principal ^{14}C-products was identified to be 4-androstene-3β,17β-diol. Verification of the identification of this product was obtained by thin-layer and gas–liquid chromatography, recrystallization, and mass spectrometry.

INTRODUCTION

The protozoan *Tetrahymena pyriformis* does not have an absolute nutritional requirement for steroids, and cannot synthesize them *de novo* [1]. Certain steroids such as progesterone or 11-deoxycorticosterone have been shown to inhibit the growth rate of *T. pyriformis* [2–7]; whereas, sterols such as stigmasterol slightly elevate the growth rate [1–5]. Cholesterol can partially reverse the growth inhibition caused not only by other steroids, but also by other inhibitors such as triparanol and dinitrophenol [1–5, 8–11]. Incubation of *T. pyroformis* with selected sterols results in the introduction of 5-ene, 7-ene, and 22-ene double bonds, and the removal of ethyl groups, but not methyl groups, from C-24 [1, 12–16].

Our objective was to determine if a steroid that was potentially growth inhibitory to *T. pyriformis* would be metabolized by this protozoan.

EXPERIMENTAL

Growth and maintenance of T. pyriformis. *T. pyriformis*, mating type II, variety 1 and/or strain W were used in all incubations. Cultures were maintained and grown at 28°C on a medium consisting of (g/l) bacteriological peptone, 10; yeast extract, 2; K_2HPO_4, 1; $MgSO_4 . 7H_2O$, 0·5; CH_3COOK, 1; and glucose, 10 (added after autoclaving). The pH was adjusted to 7·0–7·3.

Fermentation of T. pyriformis *cultures with* ^{14}*C-labeled testosterone and recovery of the radioactive metabolites.* [4-^{14}C]-Testosterone (58·8 mCi/mmol) was obtained from New England Nuclear. It migrated as a single component on each of three solvent systems before and after autoclaving in propylene glycol.

A 1×10^{-6} M to $2{\cdot}4 \times 10^{-6}$ M concentration range for testosterone was selected because Hamana and Iwai [7] found that the addition to logarithmically growing cells of 2×10^{-4} M concentrations of 4-androstene-3,17-dione, testosterone, and corticosterone slightly inhibited the growth of *T. pyriformis* cultures, and that concentrations of progesterone above 8×10^{-5} M killed the cells. Absorbance readings of the cultures, taken when the 24 h culture was added to the substrate and again 24 h later, indicated that testosterone did not cause any significant growth inhibition at the concentrations employed.

[4-^{14}C]-Labeled testosterone (0·33 to 1 μCi) was incubated in duplicate in 0·1 ml of propylene glycol and autoclaved at 1·05 kg/cm.^{2} pressure for 20 min. The *T. pyriformis* culture was prepared for incubation by transferring a 10% aliquot to 5 ml of fresh medium 24 h before addition to the steroid. An aliquot (4 ml) of the 5 ml culture was then transferred aseptically to the prepared substrate tube. Cultures were incubated at an angle of 15° and a temperature of 28°C. Two ml aliquots were aseptically removed for analysis after 24 and 48 h. Five drops of 1 M HCl were added to each of the 24 and 48 h incubation samples. After centrifugation for 15 min at 17,000 *g*, the supernatants were removed to separate tubes, and extracted four times with 3 ml portions of ethyl acetate. The pellets were extracted three times with 2 ml portions of 95% ethanol. The medium and pellet extracts were separately evaporated *in vacuo*. Quantitation of the radioactivity for this study was done with a Beckman liquid scintillation system LS-133, using the pre-set channel for ^{14}C and PPO-POPOP liquid scintillator.

Analysis of testosterone metabolites. The technique for the preparation of silicic acid columns, and the automated system for gradient elution chromatography have been previously described [17–19]. Reference testosterone (300 μg) was added to each extract to serve as a marker. A linear gradient program of 1% increase of dichloromethane in petroleum ether per tube from 0% to 100% was used to elute

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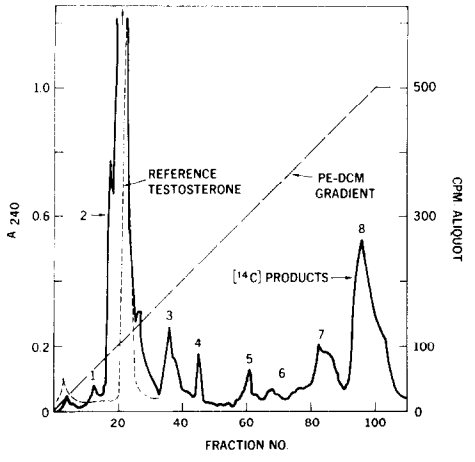

Fig. 1. Column chromatography of [^{14}C]-products in extract of spent medium from 48 h incubation of [4-^{14}C]-testosterone with *T. pyriformis*, Strain W.

The extract of the spent medium was concentrated and resolved by partition chromatography on a silicic acid column (60% aqueous, V/W) by development with a PE:DCM gradient.

the labeled products from the column (see Fig. 1). The positions of the radioactive peaks eluted were determined by counting aliquots of each fraction, and the marker was located by measuring the absorbance of the fractions at 240 nm. The column fractions comprising each of the radioactive peaks were pooled, concentrated *in vacuo*, and chromatographed in several thin-layer chromatographic solvent systems [20] on silica gel G, and the migration rate of the radioactive products relative to the mobility of progesterone (R_p) was determined. A computer program functioning as a text editor and remote job entry facility was used [20] to compare the R_p values of the products to the R_p values of 107 steroid standards. A tentative identification was noted when a labeled product repeatedly co-chromatographed with reference standards. Additional evidence for the identification of a ^{14}C-product was provided if, after being subjected to acetylation, it migrated with acetylated reference standard. Verification of identity was obtained by recrystallization of the ^{14}C-product with reference steroid, without loss of specific activity, from three different solvent systems.

Large scale preparation of T. pyriformis *metabolites from testosterone.*

A mixture of [4-^{14}C]-testosterone (10 μCi, 0·5 mg) and unlabeled testosterone (31·67 mg) was the substrate for a 5·5 l fermentation of *T. pyriformis*, strain W. The autoclaved steroid mixture was added to the 24 h culture in a Fermentation Design fermentor at a temperature of 28°C. Aeration was provided at a flow rate of 0·2 l/min before and after the addition of the testosterone. After 48 h incubation the culture was extracted with an equal volume of ethyl acetate. The extract was concentrated and chromatographed on a silicic column as previously described. Steroid containing fractions were submitted to repetitive thin-layer chromatography to isolate the metabolite, 4-androstene-3β,17β-diol.

Gas-liquid chromatography and mass spectrometry. The t.l.c. fractions containing the presumed 3β,17β-diol from the large scale fermentation were analyzed with a Finnigan Model 5000, Gas Chromatograph equipped with a flame ionization detector. A 1·5 m, 2 mm i.d. glass column was packed with Gas Chrom Q (100–120 mesh) containing 3% SE-30 as the stationary phase. A temperature program of 100–220°C at 20°/min was utilized. The samples and standards to be chromatographed *via* g.l.c. were converted to methoxime-trimethylsilyl derivatives [21].

Mass spectrometry analyses of the t.l.c. fractions were carried out using a Finnigan Gas Chromatograph Mass Spectrometer 1015D equipped with a Model 6000 Mass Spectrometry Data System. The g.l.c. conditions that were used to obtain the retention times were maintained, and the gas liquid chromatographic peaks were analyzed by electron impact and chemical ionization procedures.

Table 1. Recovery of [^{14}C]-products from column chromatography of spent medium extract of [4-^{14}C]-testosterone fermentation

		[^{14}C] in peak	
Peak*	c.p.m.	% of spent medium extract	% of [4-^{14}C]-testosterone added to culture
1	3700	1·2	1·0
2	136,100	44·0	38·3
3	12,800	4·1	3·6
4	7100	2·3	2·0
5	5500	1·8	1·6
6	7700	2·5	2·2
7	14,100	4·6	4·0
8	38,800	12·6	10·9
Methanol wash	20,300	6·6	5·7
		79·7	69·3

* Refer to Fig. 1.

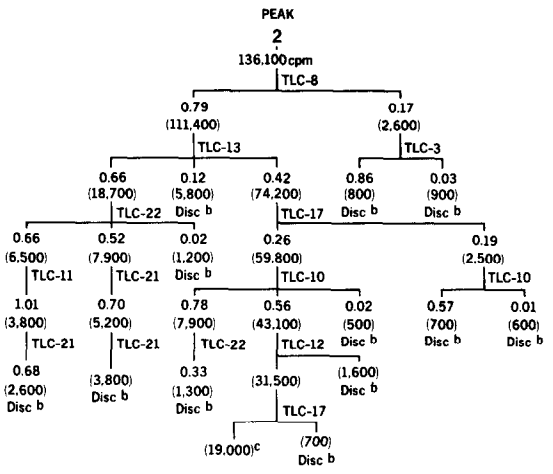

Fig. 2. Flow chart for resolution by TLC of [^{14}C]-products from [4-^{14}C]-testosterone in column peak 2 (Fig. 1).[a]

[a] Mobilities of (^{14}C)-products on the thin layer chromatograms are presented as Rp values. The cpm of each zone is shown in parentheses.

[b] Discontinued.

[c] Possible identity: 4-androstene-3β, 17β-diol or 4-androstene-3β, 17α-diol.

RESULTS

T. pyriformis converted testosterone into multiple products, none of which was identified to be unconverted substrate. The most frequently observed transformation indicated by t.l.c. analysis was the reduction of a 3-ketone to a 3β-hydroxyl. The ability of *T. pyriformis* to effect this transformation was confirmed by the identification of 4-androstene-3β,17β-diol as one of the products from the testosterone incubations. Figure 1 illustrates a typical elution pattern obtained from column chromatography of the extracts of the spent media and pellets from incubation of *T. pyriformis* with [4-^{14}C]-testosterone. The recovery of ^{14}C-products in the column peaks of Fig. 1 are summarized in Table 1.

When the major column peak of Fig. 1 was analyzed by t.l.c. the pattern of fractions depicted by the flow chart of Fig. 2 was obtained. The fraction which co-chromatographed with both 4-androstene-3β,17β-diol and 4-androstene-3β,17α-diol (as indicated in Fig. 2), was divided into two portions. Standard 4-androstene-3β,17β-diol was added to one portion, and the 17α-isomer was added to the other. Both of these standard/sample mixtures were recrystallized, and the results of these recrystallizations are presented in Table 2. Since the recrystallization of the sample with the 17β-isomer was done from three different solvents, the constant specific activity of the crystals obtained supports the identity of the transformation product of testosterone as 4-androstene-3β,17β-diol.

In order to select the concentration of testosterone for the best yield of 4-androstene-3β,17β-diol, four test tube incubations were conducted with different concentrations of unlabeled testosterone added to approximately 1 μCi of [4-^{14}C]-testosterone in each 5 ml of cultures of *T. pyriformis*, strain W. The cultures were incubated for 48 h, and extracted with equal volumes of ethyl acetate. Table 3 summarizes the data obtained when the extracts were analyzed *via* t.l.c. analysis of the column peaks. Identification of a fraction as testosterone or the β-diol was based on chromatographic behavior on thin-layer plates in a minimum of three different systems, and on t.l.c. comparison of the acetylated products with reference standard. From these results, the testosterone concentration of 2×10^{-5} M was selected for the large scale fermentation.

When the *T. pyriformis* culture from the 48 h, 5·5 liter fermentation was extracted, an 81% recovery of added radioactivity was obtained among the ^{14}C-products. The column chromatography elution pattern of the extract showed three major peaks eluting in the first 47 fractions, with a gradual tailing-off of the radioactivity to a background level at fraction 100. Table 4 indicates the recovery of added ^{14}C in each fraction. T.l.c. purification of the column peaks with four successive t.l.c. systems indicated that the greatest amount of the ^{14}C-product that co-chromatographed with standard 4-androstene-3β,17β-diol was

Table 2. Recrystallization of a [^{14}C]-product from [4-^{14}C]-testosterone with standards, 4-androstene-3β,17β-diol and 4-androstene-3β,17α-diol.
Approximately 50 mg of each standard was added to one-half of the [^{14}C]-product. Recrystallization was accomplished from the solvents indicated.

Step	Solvent	[^{14}C] Crystals c.p.m.	Weight crystals mg	Specific activity c.p.m./mg crystals
Recrystallization with 4-androstene-3β,17β-diol				
Initial		10,700	49·442	216·42
1st recrys.	methanol/water	427	2·186	195·33
2nd recrys.	acetone/water	320	1·524	209·97
3rd recrys.	dioxane/water	239	1·057	226·11
Recrystallization with 4-androstene-3β,17α-diol				
Initial		11,300	50·053	225·76
1st recrys.	methanol/water	113	1·074	105·21
2nd recrys.	acetone/water	39	2·239	17·42

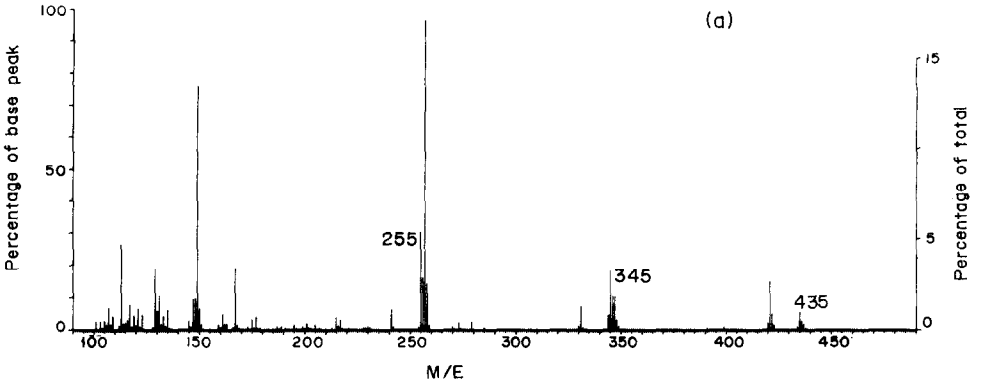

Fig. 3a. Chemical ionization spectrum of standard 4-androstene-3β,17β-diol.

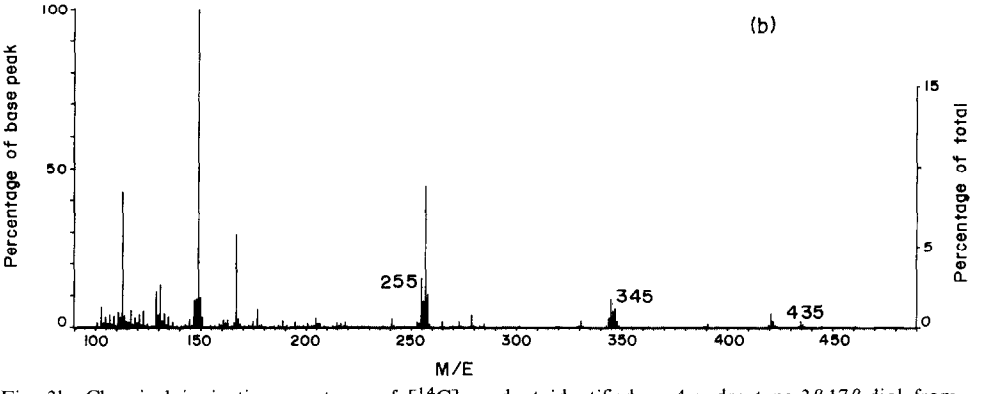

Fig. 3b. Chemical ionization spectrum of [^{14}C]-product identified as 4-androstene-3β,17β-diol from large scale fermentation of *T. pyriformis*, strain W, with [4-^{14}C]-testosterone.

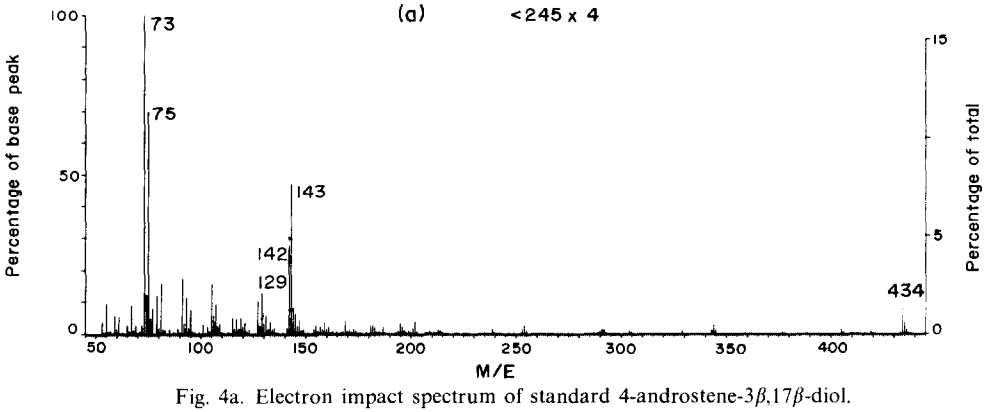

Fig. 4a. Electron impact spectrum of standard 4-androstene-3β,17β-diol.

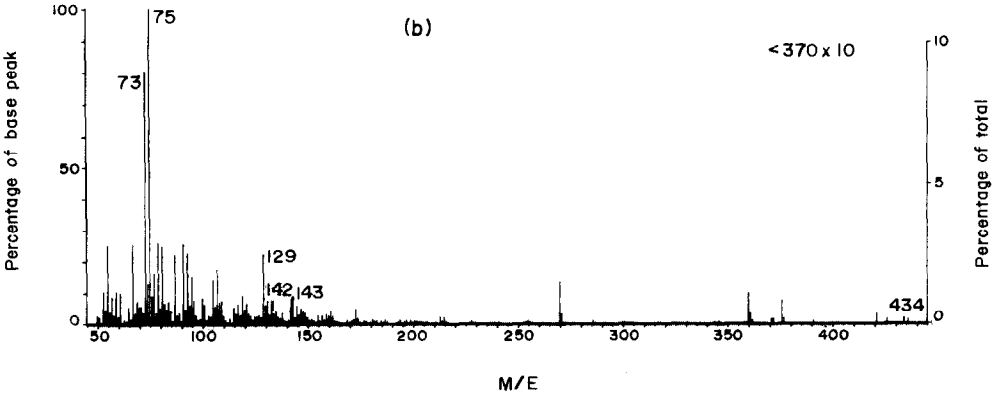

Fig. 4b. Electron impact spectrum of [^{14}C]-product identified as 4-androstene-3β,17β-diol from large scale fermentation of *T. pyriformis*, strain W, with [4-^{14}C]-testosterone.

Table 3. Incubation of *T. Pyriformis*, strain W, for 48 hr with varying concentrations of testosterone.

Concentration of testosterone	Concentration factor*	% Of added c.p.m. recovered in extract	% Of added substrate identified as testosterone	% Of added substrate identified as 4-androstene-3β,17β-diol
1×10^{-5} M	5	85·8	0·0	19·8
2×10^{-5} M	10	91·5	4·3	45·7
1×10^{-4} M	50	96·0	34·0	13·6
2×10^{-4} M	100	98·1	39·3	0·0

* Testosterone concentration of this incubation sample compared to concentration of initial testosterone incubation (Figure 1).

located in peak 3, with lesser amounts occurring in peaks 4–8. There was no ^{14}C-product that migrated like the β-diol in either peaks 1 or 2.

Each of the t.l.c. components that were tentatively identified as 4-androstene-3β,17β-diol were analyzed by g.l.c. The retention times of 4-androstene-3β,17α-diol, 4-androstene-3β,17β-diol, and testosterone were 10·0, 10·8, and 11·7 min, respectively. All of the t.l.c. fractions that appeared to contain the β-diol were analyzed by g.l.c., and were found to display a distinct peak with a retention time of either 10·8 or 10·9 min; that is, they migrated like the β-diol and not like either testosterone or the α-diol.

The presumed 3β,17β-diol from peak 3, after further purification by successive t.l.c. was analyzed by Mass Spectrometry. Both chemical ionization (Fig. 3) and electron impact (Fig. 4) spectra were obtained for the trimethylsilyl derivatives of the sample and of standard 4-androstene-3β,17β-diol. Figure 3 reveals that the reference and isolated compounds exhibited the quasi-molecular ion at *m/e* 435 $(MH)^+$. The observed $(MH-90)^+$ and $(MH-180)^+$ at *m/e* 345 and 255, respectively, were also expected from the loss of one or both trimethylsilyl ether groups (as trimethylsilanol) after the initial protonation.

The ions of interest in the electron impact spectra are the molecular ion of *m/e* 434, the base peaks at *m/e* 73 and 75, and the fragments at *m/e* 129, 142, and 143. Although the standard and sample spectra are not exactly alike, the occurrence of these *m/e* fragments in both spectra provide further proof of the 4-ene-3β-diol structure. Specifically, the base peak of either *m/e* 73 or 75 eliminates the structures of 4-androstene-3α,17β-diol and 5-androstene-3β,17β-diol, since the trimethylsilyl ethers of these compounds would have base peaks at *m/e* 143 and 129, respectively [22]. Although the spectra do show a *m/e* of 129, it is not the base peak, and the $(M-129)^+$ fragment, which would attest to the presence of a 5-ene-3β-hydroxy structure in the parent steroid [117], is absent. The *m/e* 129 in the spectra of 4-androstene-3β,17β-diol trimethylsilyl ether probably represents a fragment derived from C-15 to C-17 with substituents $[CH_2\text{-}CH_2CHO\text{-}Si(CH_3)_3 = 130]$, less one hydrogen atom [23]. The *m/e* fragments at 143 and 142 are also characteristic of 3-enoltrimethylsilyl ethers, and are reported to be derived from C-1 to C-4 with substituents $[CH_2\text{-}CH_2\text{-}CHO\text{-}Si(CH_3)_3\text{-}CH = 143]$ and this same fragment less one hydrogen atom, respectively [24]. The mass spectrum of 4-androstene-3β,17β-diol silyl ether has been published by Neville and Engel [25].

Table 4. Recovery of [^{14}C]-products from column chromatography of the extract from the large scale [4-^{14}C]-testosterone fermentation
[4-^{14}C]-Testosterone (10 μCi; 2×10^{-5} M) was incubated at 28°C for 48 h with 5·5 l of *T. pyriformis*, strain W. The culture was extracted and analyzed as described in the Materials and Methods.

		[^{14}C] in peak	
Peak	c.p.m. $\times 10^{-4}$	% of Extract	% of [4-^{14}C]-Testosterone added to culture
1	270	20	16
2	176	13	10
3	245	18	15
4	51	4	3
5	75	6	4
6	39	3	2
7	27	2	2
8	9	1	1
Methanol wash	3	0	0
		67	53

DISCUSSION

The reduction of testosterone to 4-androstene-3β,17β-diol by *T. pyriformis* is of interest, since only dehydrogenation and removal of a C-24 ethyl group have been reported for the transformation of sterols by *T. pyriformis* [1, 12].

The conversion of a 4-ene-3-ketosteroid to a 4-ene-3α- or 4-ene-3β-hydroxy-steroid has been reported to occur in several tissue preparations. Farnsworth *et al.* tentatively identified 4-androstene-3ε,17β-diol after incubation of prostate mince with testosterone [26], and Levy *et al.* identified 3α,17-dihydroxy-4-pregnen-20-one as a metabolite of 17-hydroxy-4-pregnene-3,20-dione, which was perfused through bovine adrenals or ovaries [27].

Incubation of supernatant fractions from rabbit skeletal muscle [28], rat kidney [29], and sheep adrenals [30, 31] with 4-androstene-3,17-dione yielded 3β-hydroxy-4-androsten-17-one. Both 3α,11β-dihydroxy-4-androsten-17-one and 3β,11β-dihydroxy-4-androsten-17-one [32] have been identified in human urine. However, the formation of 4-ene-3α- or 4-ene-3β-hydroxysteroids represents an unusual metabolism of 4-ene-3-ketosteroids, in that it seems to occur only in the absence of ring reductases, e.g., in tissues such as kidney, skeletal muscle, or human fetal liver, which have a low activity of ring-reductase [22, 28, 29]. In the presence of ring-reductases, e.g., in a rat liver system, 4-ene-3-ketosteroids are first reduced to the 4,5-dihydro-3-keto compounds, which are then reduced further to 3-hydroxysteroids [33, 34]. In a liver microsomal preparation, testosterone and 17-epitestosterone were metabolized to 4-androstene-3α (3β), 17α (17β) -diols [35, 36].

3β-Hydroxy-4-androsten-17-one and 3β-hydroxy-5-androsten-17-one have been identified as products from the incubation of 4-androstene-3,17-dione with sheep adrenal preparations [37], and it was thought that the 4-ene-3β-ol was an intermediate in the reversible 5-ene-3β-ol to 4-ene-3-ketone pathway, which is prominent in adrenal, testicular, and ovarian tissue. However, it was later established [30] that the 5-ene-3β-ol to 4-ene-3-ketone conversion (or the reverse) occurs *via* the 5-ene-3-ketone intermediate, and therefore the occurrence of 4-ene-3-hydroxysteroids in endocrine tissue remains unexplained.

Yeast has been the preferred organism for the microbial reduction of carbonyl compounds, since it usually does not cause other transformations simultaneously [38]. The reduction of a carbonyl at C-3 in saturated steroids by *S. cerevisiae* has been frequently reported [38], but the carbonyl at C-3 of 4-ene-3-ketosteroids resists reduction by yeast [31, 38]. However, Schubert *et al.* reported the conversion of testosterone and 17α-methyl-testosterone to 3α(3β)-hydroxy-5α-androstanes by *Rhodotorula glutinis* [39], and Butenandt *et al.* [40] have reported the reduction of 5α-1-androstene-3,17-dione to 5α-androstene-3β,17β-diol by *S. cerevisiae*. *Clostridium paraputrificum* is capable of anaerobically converting 4-ene-3-ketosteroids to 3β-hydroxy-5β-steroids [41].

The β-hydroxysteroid dehydrogenase (β-HSDH) of *Pseudomonas testosteroni* is an NAD-linked enzyme that catalyzes the reversible oxidation of 3β- and 17β-hydroxyl groups, but in the absence of electron withdrawing groups (e.g. halogens at positions 2, 4, and 6), the equilibrium favors oxidation rather than reduction [42]. This observation supports the theory that the hydride ion transfer from NADH to the carbonyl is the rate-controlling step for the reduction reaction. That is, decreasing the electron density at the carbonyl group by the presence of highly electronegative species at positions where they may by induction withdraw electrons from the carbonyl, should increase the rate of the reduction process, if the hydride ion transfer is rate-limiting. Ringold *et al.* [42] have demonstrated that cell free extracts of *Ps. testosteroni* reduce testosterone, substituted at the 2α-, 4-, 6α- or 6β-positions with fluorine, or at the 4-position with chlorine, to a mixture of the corresponding 3α- and 3β-ols; whereas, reduction of unsubstituted testosterone at the 3-carbonyl did not occur to any significant degree. 4-Chlorotestosterone was converted to 4-chloro-4-androstene-3α,17β-diol and 4-chloro-4-androstene-3β,17β-diol in human liver slices *in vitro* [43].

It would be of interest to determine if the β-HSDH of *T. pyriformis* has similar properties to that of *Ps. testosteroni*.

Two protozoan species of the genus *Trichomonas* reduced the carbonyl at C-17 to a 17β-ol in C_{18} and C_{19} steroids, but did not reduce the carbonyl at C-3 in a 4-pregnen-3-one [44].

T. pyriformis, strain W, has also been reported to reduce the conjugated ketone of an aflatoxin to a 3β-ol [45].

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