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THE CONVERSION OF PROGESTERONE TO PREGNENOLONE BY *TETRAHYMENA PYRIFORMIS*

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SUMMARY

[^{14}C]-Pregnenolone was the major product when [4-^{14}C]-progesterone was incubated with cultures of *Tetrahymena pyriformis*, strain W. The identification of [^{14}C]-pregnenolone was verified by thin-layer and gas chromatography, recrystallization, and mass spectrometry.

INTRODUCTION

We have recently reported the transformation of testosterone to 4-androstene-3β,17β-diol by *Tetrahymena pyriformis* [1]. The conversion of a 4-ene-3-ketosteroid to a 4-ene-3-hydroxysteroid is not without precedent with *in vitro* systems [2-18], and is considered to be due to some reversibility in the oxidation of 3-hydroxysteroids to 3-ketosteroids, which is catalyzed by 3β(α)-hydroxysteroid dehydrogenases. Both 3α,11β-dihydroxy-4-androsten-17-one and 3β,11β-dihydroxy-4-androsten-17-one have been identified in human urine [19].

Since the reduction of the 3-carbonyl of testosterone by *T. pyriformis* occurred with whole cells, and 4-androstene-3β.17β-diol was the major product of the fermentation, it was our objective to determine if this unusual transformation could be repeated using another 4-ene-3-ketosteroid as substrate. Progesterone was selected because it is known to have a greater growth inhibitory capacity than testosterone, when added to cultures of *T. pyriformis* [20, 21].

EXPERIMENTAL

Fermentation of T. pyriformis *cultures with* [4-^{14}C]-*progesterone.* Cultures of *T. pyriformis* strain W were maintained at 28°C on a medium previously described [1]. [4-^{14}C]-Progesterone (56.0 mCi/mmol, New England Nuclear Corporation) was shown to be homogeneous by t.l.c. in three solvent systems before and after autoclaving in propylene glycol. Approximately 0.4 μCi of [4-^{14}C]-progesterone was autoclaved in 0.1 ml propylene glycol at 1.5 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. A 4 ml aliquot of the 24 h culture was transferred aseptically to the prepared substrate tube. Duplicate cultures were incubated at a temperature of 28 C. and 2 ml aliquots were removed after 48 h. The cells were pelleted by centrifugation and the spent medium was extracted with ethyl acetate. To verify that the radioactive products obtained were a result of a transformation by *Tetrahymena pyriformis* duplicate tubes of media plus [4-^{14}C]-progesterone were also incubated. Chromatography of this extracted media in three different t.l.c. systems yielded only one peak which migrated with standard progesterone. Quantitation of the radioactivity in the spent medium extract 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 progesterone metabolites. The procedure for chromatography of the extract on a silicic acid column using an automated system for gradient elution has been previously described [1]. The column fractions comprising each of the radioactive peaks were pooled, concentrated *in vacuo*, and analyzed by chromatography in several thin-layer chromatographic systems [1]. Additional evidence for the identification of a ^{14}C-product was provided by gas liquid chromatography with a Finnegan Model 5000 Gas Chromatograph equipped with a flame ionization detector and a Radioactive Monitoring System (Searle Analytic Inc., Model 7357). A 1.5 m, 2 mm id glass column was packed with Gas Chrom Q (100–120 mesh) containing 1.5% OV-1 as the stationary phase. A temperature program of 150°–200°C at 20°/min and a flow rate of 40 ml/min of argon were utilized. The samples and standards to be chromatographed via g.l.c. were converted to methoxime-trimethylsilyl derivatives [22].

Large scale preparation of T. pyriformis *metabolites from progesterone.* Unlabeled progesterone and [4-^{14}C]-progesterone were mixed as the substrate for an 8 l. fermentation of *T. pyriformis*. The concentration of progesterone (2×10^{-6}M) in the culture was selected to repeat the condition of the test tube

incubation, and avoid the potential growth inhibitory effect of progesterone [21]. The autoclaved steroid mixture was added to the 24 h culture in four 3 l. flasks. Aeration was provided by shaking, and the temperature was 28°C. After 48 h the culture was extracted with ethyl acetate, and the extract was analyzed via column, thin-layer, and gas liquid chromatography [1]. Mass spectrometry analyses of the t.l.c. fractions was done on a Finnegan Gas Chromatograph Mass Spectrometer 1015D, and the chromatographic peaks were analyzed by electron impact and chemical ionization procedures. Verification of the identity of the [^{14}C]-product was also obtained by recrystallization with standard pregnenolone to a constant specific acitivity.

RESULTS

The major [^{14}C]-product isolated from the spent medium of the 4 ml fermentation eluted from the silicic acid column in the same fractions as the reference progesterone which was added as a marker. See Fig. 1 column peak 3. When column peak 3 was analyzed by t.l.c., the fractions indicated in the flow chart of Fig. 2 were obtained. The major t.l.c. fraction of $R_p = 0.86$ co-chromatographed in five different t.l.c. systems with several steroid standards; one of which was pregnenolone. When this [^{14}C]-product was analyzed by g.l.c. it had a retention time that coincided exactly with pregnenolone and differed appreciably from the retention time of the other steroid standards. Quantitatively, this product represented 26% of the [4-^{14}C]-progesterone added as substrate, and 43% of the [^{14}C]-products isolated in the extract. The identification of the other [^{14}C]-products was not pursued.

When the extract from the 8 l. fermentation was chromatographed on the silicic acid column, 85% of the radioactivity was eluted in the first ten fractions. The residue which was obtained from evaporation of these fractions was a viscous brown oil. A series of preparative thin-layer systems was used to achieve a separation of 63% of the radioactivity from the oil. Several of the cleaner radioactive t.l.c. fractions were analyzed by g.l.c., and the radioactivity chromatographed as a single sharp peak with the retention time of standard pregnenolone (7.2 min). The retention time of progesterone was 8.5 min. The amount of [^{14}C]-product that co-chromatographed with standard pregnenolone on t.l.c. and g.l.c. represented 51% of the [^{14}C]-progesterone added as substrate and 68% of the isolated [^{14}C]-products.

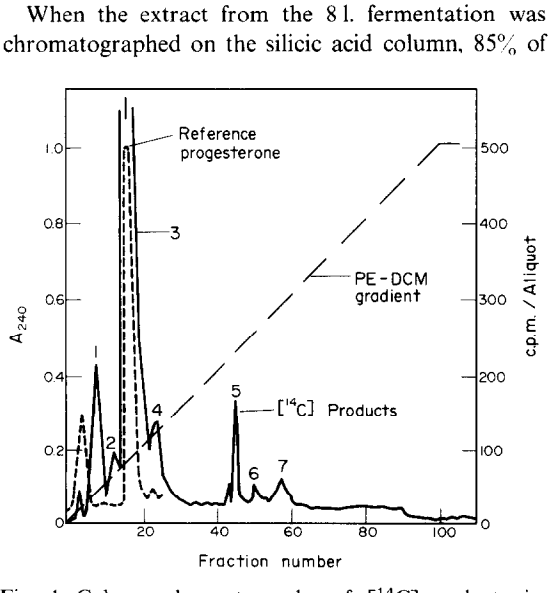

Fig. 1. Column chromatography of [^{14}C]-products in extract of spent medium from 48 h incubation of [4-^{14}C]-progesterone with *T. pyriformis*, Strain W. The extract of the spent medium was concentrated and resolved by partition chromatography on a silicic acid column (60% aqueous V/W) by development with a PE:DCM gradient.

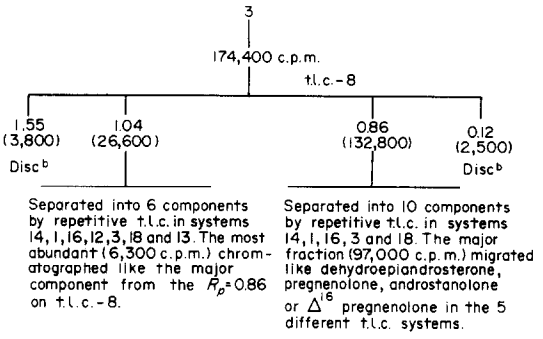

Fig. 2. Flow chart for resolution by t.l.c. of [^{14}C]-products from [4-^{14}C]-progesterone in column fraction 3 (Fig. 1). [a]Compositions of the t.l.c. systems are given in Reference [41]. Mobilities of [^{14}C]-products on the thin-layer chromatograms are presented as R_p values, which are the migration rates of the radioactive products relative to the mobility of progesterone. The c.p.m. of each zone is shown in parentheses. [b]Discontinued.

The major t.l.c. fraction which was obtained by preparative chromatography of the major column peak in TLC systems 14, 10 and 1 [41], and was still contaminated with brown oil, was selected for purification by recrystallization. Standard pregnenolone was mixed with a portion of the radioactive product (the remainder was used for mass spectrometry), and the mixture was recrystallized from several different solvent systems until purification was indicated by a stabilization in the S.A. of the crystals (Table 1, 1st to 4th recrys.). The relatively constant values of the specific activities for the last four recrystallizations indicates that 37% of the t.l.c. fraction was [^{14}C]-pregnenolone.

The second column peak which was actually a shoulder of the major column peak was selected for analysis by mass spectrometry because it contained less of the brown oil than the major peak. Preparative t.l.c. of this shoulder peak in systems 14, 10 and 1 [41], yielded a sample that gave the spectra of Figs 3b and 4b. The spectra of standard pregnenolone are given in Figs 3a and 4a for comparison. Figure 4c is an electron impact spectrum of the t.l.c. fraction which was selected for purification by recrystallization.

Table 1. Recrystallization of [^{14}C]-product from [4-^{14}C-]-progesterone with standard pregnenolone

Step	Solvent	[^{14}C]-Crystals c.p.m.	Weight crystals mg	S.A.c.p.m./mg crystals
Initial		24,100	74.883	321.84
1st recrys.	Methanol/water	328	1.554	211.07
2nd recrys.	Acetone/water	389	2.295	169.50
3rd recrys.	Dioxane/water	260	1.732	150.12
4th recrys.	Cyclohexane	195	1.630	119.63
5th recrys.	Methanol	206	1.746	117.98
6th recrys.	Isooctane	115	1.068	107.68
7th recrys.	Cyclohexane	233	1.995	116.79
8th recrys.	Isooctane	302	2.531	119.32

Approximately 75 mg of pregnenolone was added to the [^{14}C]-product. Recrystallization was accomplished from the solvent indicated.

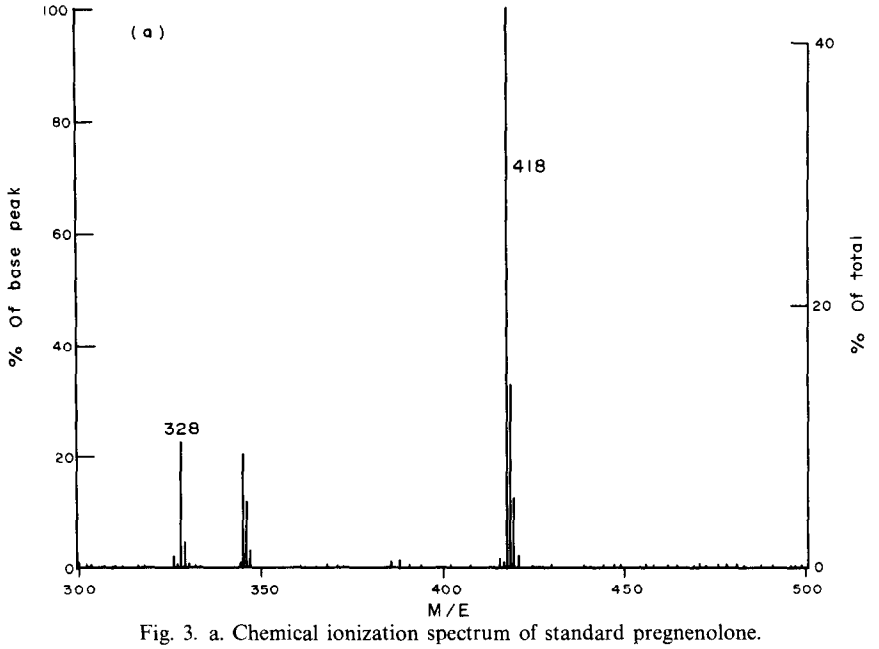

Fig. 3. a. Chemical ionization spectrum of standard pregnenolone.

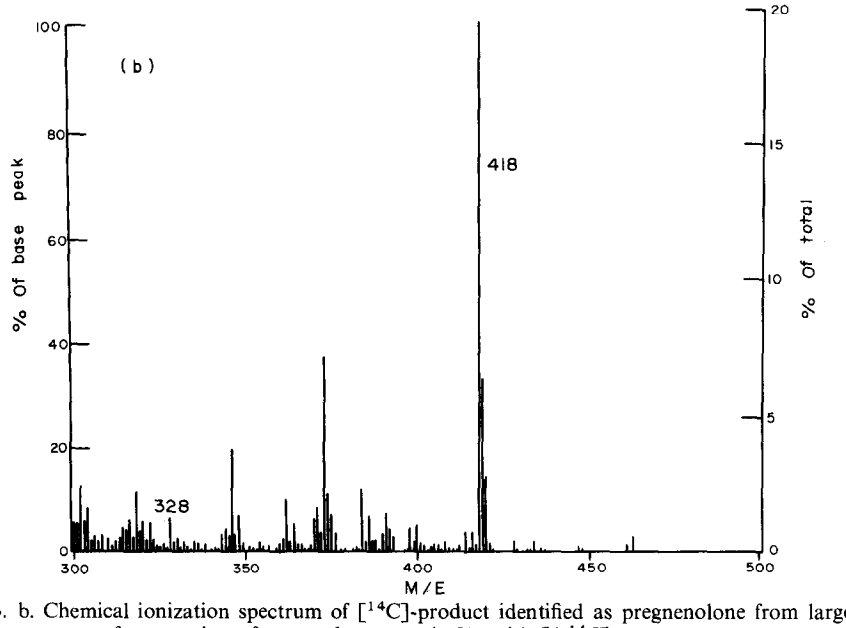

Fig. 3. b. Chemical ionization spectrum of [^{14}C]-product identified as pregnenolone from large scale fermentation of *T. pyriformis*, strain W, with [4-^{14}C]-progesterone.

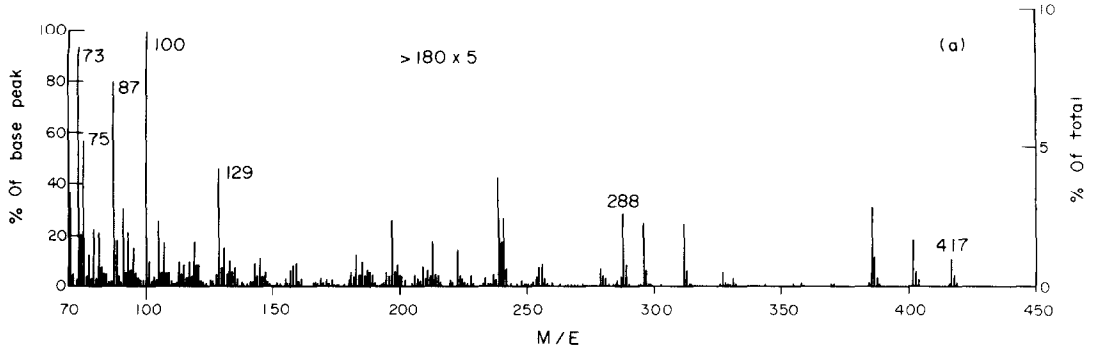

Fig. 4. a. Electron impact spectrum of standard pregnenolone.

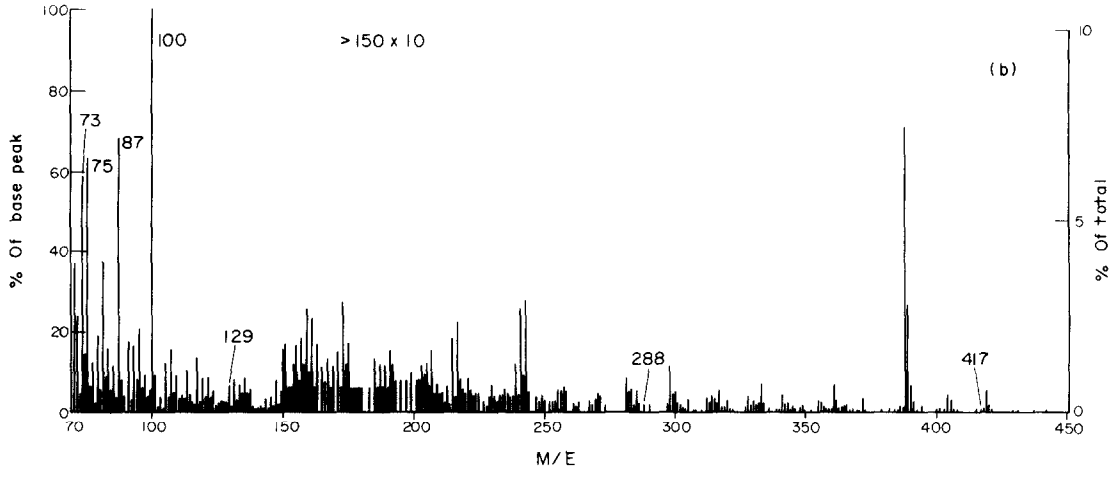

Fig. 4. b. Electron impact spectrum [^{14}C]-product identified as pregnenolone from large scale fermentation of *T. pyriformis*, strain W, with [4-^{14}C]-progesterone.

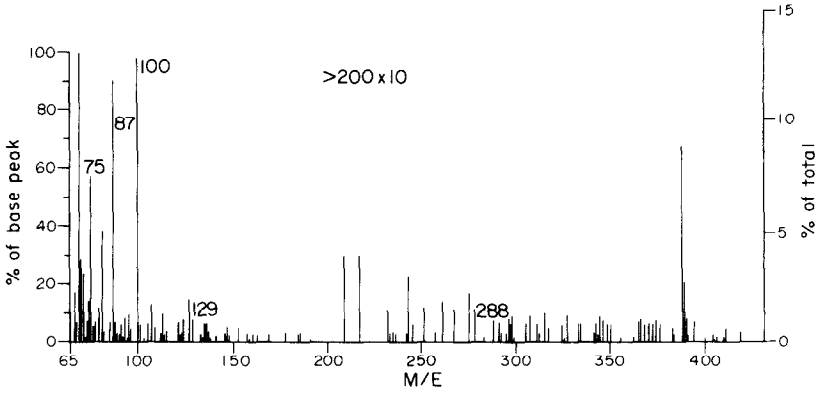

Fig. 4c. Electron impact spectrum of [^{14}C]-product identified as pregnenolone by recrystallization.

The chemical ionization spectra of the sample and of standard pregnenolone (Figs 3a and 3b) exhibited the quasi-molecular ion of *m/e* 418 (MH^+), and the $(MH\text{-}90)^+$ at *m/e* 328, which is expected from the loss of the trimethyl ether group (as trimethylsilanol) from C-3 after the initial protonation. The quasi-molecular ion at *m/e* 418 was the base peak in both standard and sample spectra.

The base peak of the electron impact spectra of the samples and standard pregnenolone (Figs 4a, 4b and 4c) was at *m/e* 100, and is reportedly composed of C-16/C-17 with substituents and one additional hydrogen atom [23]. Aside from the molecular ion at *m/e* 417 which appears as a small fragment in the standard and the Fig. 4b sample spectra, the major ions of interest were the *m/e* at 129 and 288 (M-129). The appearance of these two ions in the sample spectra attests to the presence of 5-ene-3β-hydroxy group in the steroid [24]. It is the location of the double bond at C-5 which directs the fragmentation to produce the ion composed of C-1 to C-3 with substituents less one hydrogen atom (*m/e* 129) [25–26]). The accompanying complementary ion at M-129 is characteristic of the 5-ene-3-trimethylsilyloxy group [25]. Other major ions which appear in both the standard and sample spectra are those at *m/e* 73.

75 and 87. The latter consists of C-17 with substituents and two additional hydrogen atoms [23]. TMSI ethers of 4-ene-3β-hydroxysteroids give abundant ions of *m/e* 142 and 143 [27–28]. These ions were not significant in the sample spectra. Comparison of the spectra of Figs 4b and 4c indicates the difference in fragmentation patterns due to impurities that are co-chromatographing with the radioactive product. Thus it is the pattern of fragments from *m/e* 70–100 of the Fig. 4b spectrum and the *m/e* fragments 129 and 288 of the Fig. 4c spectrum which were taken as further proof of the 5-ene-3β-diol structure.

The [^{14}C]-product identified as pregnenolone was isolated from the spent medium of the 4 ml fermentation and an extract of the spent medium plus cells of the 8 l. fermentation. Therefore, nothing can be said as to the distribution of this metabolite; that is, in the protozoa *versus* in the medium. However, the control study confirms that this transformation only occurs in the presence of *Tetrahymena pyriformis*.

DISCUSSION

The transformation of progesterone to pregnenolone by *T. pyriformis* involves the conversion of a 4-ene-3-ketosteroid to a 5-ene-3β-hydroxysteroid. Although the reduction of the 3-carbonyl to the 3β-hydroxyl was expected from our previous study [1], the isomerization of the double bond from the C_4-C_5 to the C_5-C_6 position represents a new steroid transformation by this protozoan.

The conversion of a 5-ene-3β-hydroxysteroid to a 4-ene-3-ketosteroid has been described in many enzyme systems of animal, vegetable and bacterial origin [29], and is known to be an important step in the synthesis of biologically active steroid hormones [30]. This conversion is now believed to consist of two separate enzymatic steps [30–32]: a reversible oxidation of the hydroxyl group to a ketone catalyzed by a 3β-hydroxysteroid dehydrogenase [2–18], followed by the shift of the C_5-C_6 double bond to the C_4-C_5 position [31, 33]. The isomerization was thought to be irreversible until a compound behaving like pregnenolone was isolated from rat plasma [34]. Conversion of progesterone to pregnenolone has now been demonstrated by enzyme preparations from rat ovary [35] and rabbit testes [36]. The reduction of androstenedione to dehydroepiandrosterone has been shown to occur with an acetone powder of sheep adrenal microsomes [6, 11]. However, it has been established that a significant reversibility in the isomerase reaction (i.e. 4-ene $\rightarrow$ 5-ene) only occurs if it is coupled to a hydroxysteroid dehydrogenase reaction [16]. The pathway has now been confirmed to be 4-ene-3-ketone $\leftrightarrows$ 5-ene-3-ketone $\rightleftarrows$ 5-ene-3β-hydroxy- with the verification of the 5-ene-3-ketone intermediate [16, 37].

The transformation of progesterone to pregnenolone by *T. pyriformis* is the first reversibility of this pathway to be demonstrated with an intact organism. Whether the isomerase and dehydrogenase activities of *T. pyriformis* are associated with separate proteins as in the bacterium *Pseudomonas testosteroni* [38–39] is unknown. To date, these enzymes from mammalian sources have not been separated [40].

When testosterone (a C-19 steroid) was incubated with cultures of *T. pyriformis*, the major product isolated from the spent medium was 4-androstene-3β,17β-diol [1]; that is, the double bond at C_4-C_5 was not isomerized. It remains to be determined whether isomerization of the double bond in progesterone is due to a preference of the *T. pyriformis* isomerase for a C-21 steroid.

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