MIGRATION OF THE C-25 HYDROGEN OF CLIONASTEROL TO THE C-24 POSITION DURING DEALKYLATION BY THE PROTOZOAN, *TETRAHYMENA PYRIFORMIS*

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1. Introduction

The ciliate protozoan *Tetrahymena pyriformis* cannot synthesise sterols, but carries out a number of transformations of sterols present in its growth medium. These transformations include the insertion of $\Delta 5$, $\Delta 7$ and $\Delta 22$ double bonds into the sterol [1], and the removal of 24 R and 24 S ethyl, but not methyl or ethylidene, groups from C-24 of phytosterols (dealkylation) [2].

A number of insects can also dealkylate phytosterols, although in this case the range of C-24 substituents which can be removed is much greater, since methyl, ethyl and ethylidene groups at C-24 can be removed. Evidence exists for the operation of the pathway (fig.1) sitosterol (I), → fucosterol (II) → fucosterol 24,28 epoxide (III) → desmosterol (IV) → cholesterol (V) for sitosterol dealkylation in insects [3]. We have previously shown that dealkylation of 28-isofucosterol (VI) by the insect *Tenebrio molitor* involves retention of the C-25 hydrogen atom [4]. The implication of desmosterol in the pathway requires this hydrogen to migrate if it is to be retained, and its migration to C-24 during dealkylation of clionasterol (VII) has been demonstrated [5].

In contrast to insects, *Tetrahymena pyriformis* does not dealkylate the 24-ethylidene sterols fucosterol and 28-isofucosterol although these sterols are dehydrogenated by the protozoan. A dealkylation mechanism not involving the intermediacy of a 24-ethylidene sterol has been proposed for *Tetrahymena*. This involves 29-hydroxylation followed by phosphorylation of this hydroxyl group and hydride ion attack at C-24, eliminating ethylene and giving cholesterol. This mechanism would imply retention of the C-25 hydrogen atom without migration [6].

We have administered [25-^{3}H, 26-^{14}C]clionasterol to *Tetrahymena* and shown that in this organism, as in insects, the C-25 hydrogen migrates to C-24 during dealkylation.

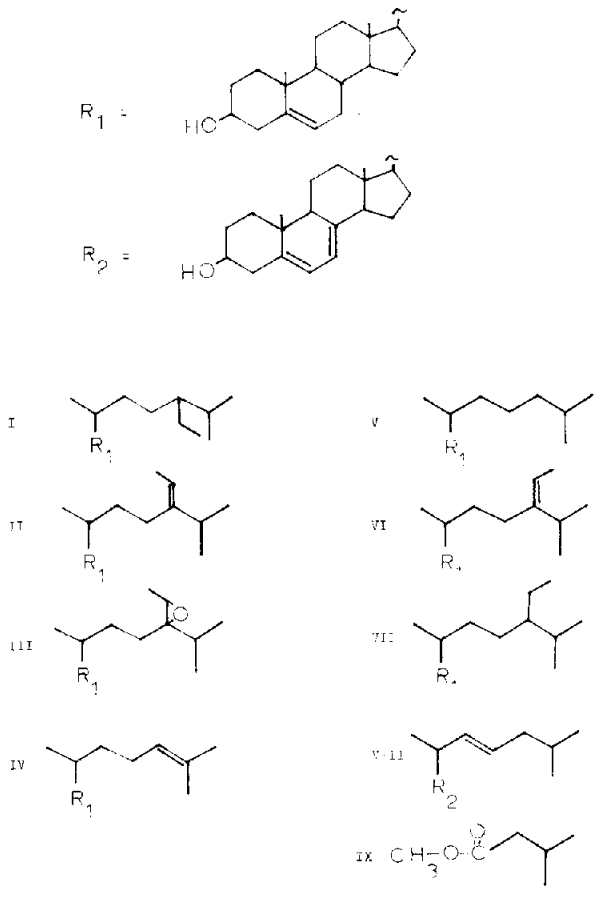

Fig.1. Structures of sterols and derivatives.

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2. Methods

Tetrahymena pyriformis, strain W, was obtained from the Culture Centre for Algae and Protozoa, Cambridge, England, and was maintained on a medium containing proteose peptone (1% w/v), glucose (1% w/v) and yeast extract (0·25% w/v). Cells were grown at 28°C with vigorous aeration and were harvested by centrifugation. Sterols were added to the medium dissolved in a minimum volume of ethanol. [25-^{3}H] and [26-^{14}C]clionasterol were synthesised as previously described [5].

Sterols were isolated from the harvested protozoan by saponification under nitrogen and extraction of the nonsaponifiable lipid fraction. This fraction was subjected to column chromatography on Brockmann Grade III neutral alumina developed with increasing concentrations of diethyl ether in light petroleum (b.p. 40–60°C). The sterol-containing fraction (eluted by 30% ether) was further fractionated by t.l.c. on silica gel GF_{254} developed with chloroform and the UV absorbing sterol band was eluted from the plate. The crude sterol fraction was separated on the basis of number and position of double bonds by t.l.c. on 10% (w/w) silver nitrate-impregnated silica gel GF_{254} developed with chloroform: ether: glacial acetic acid (97:2·5:0·5, by vol.) The major band (R_f 0·13, sterol Δ 5,7,22 trienes) and a band at R_f 0·17 (sterol Δ 5,7 dienes) was uv absorbing, while a further band at R_f 0·32 (Δ5 and Δ5,22 sterols) was visualised by spraying the plate with 10% Rhodamine 6G in acetone.

When the protozoan was grown in the presence of cholesterol (15 mg/litre of medium) the material eluted from the major band was crystallised from chloroform–methanol to give white crystals of cholesta-5,7,22-trien-3β-ol (VIII) which was fully characterised by NMR and mass spectrometry.

Dealkylation experiments involved the addition of [25-^{3}H, 26-^{14}C]clionasterol (6 mg) to 400 ml of medium. The sterol triene band was isolated as described above, and a portion of this material was subjected to preparative g.l.c. on 3% OV-17 to separate the C_{27} and C_{29} sterol trienes. The cholesta-5,7,22-trien-3β-ol thus isolated was further purified by t.l.c. after addition of carrier material and was recrystallised from chloroform–methanol to constant specific radioactivity. An aliquot of the administered clionasterol was similarly recrystallised.

Location of the tritium in the doubly labelled cholesta-5,7,22-trien-3β-ol was carried out essentially by the method of Watkinson et al. [7].

3. Results and discussion

The distribution of radioactivity in the sterol fractions of *Tetrahymena pyriformis* after incubation with [25-^{3}H, 26-^{14}C]clionasterol (VII, 55 μCi of ^{3}H, 10 μCi of ^{14}C) is shown in table 1. Approximately 16% of the clionasterol taken up by the cells has undergone dealkylation and dehydrogenation to give cholesta-5,7,22-trien-3β-ol (VIII). The ^{3}H/^{14}C ratio of this sterol is compared with that of the original clionasterol in table 2, and it is clear that the C-25 hydrogen atom has been retained during dealkylation. Location of the tritium radioactivity in cholesta-5,7,22-trien-3β-ol was carried out by permanganateperiodate oxidation of the sterol to yield isovaleric acid as the side chain fragment containing carbon atoms 23,24,25,26 and 27 of the original sterol. After addition of carrier isovaleric acid, this was methylated and purified by distillation. Base equilibration of the radioactive methyl isovalerate (IX) with sodium methoxide in methanol resulted in the loss of over 90% of the tritium (table 2), thus demonstrating that in the original cholesta-5,7,22-trien-3β-ol, the tritium was located at C-24. Therefore, dealkylation of clionasterol in *Tetrahymena pyriformis* involves migration of the C-25 hydrogen of the substrate to C-24 in the product. This migration also occurs

Table 1
Distribution of radioactivity in the sterols of *Tetrahymena pyriformis* after incubation with [25-^{3}H, 26-^{14}C]clionasterol (10 μCi of ^{14}C)

Sterol fraction	Radioactivity (d.p.m. ^{14}C)	Relative %
Δ5 and Δ5,22 (C_{27} and C_{29})	$1·76 \times 10^6$	67
Δ5, 7 (C_{27} and C_{29})	$1·74 \times 10^5$	7
Δ5,7,22 (C_{27})	$4·13 \times 10^5$	16
Δ 5,7,22 (C_{29})	$2·75 \times 10^5$	10

Table 2
Analysis of cholesta-5,7,22-trien-3β-ol biosynthesised from [25-^{3}H, 26-^{14}C]clionasterol by *Tetrahymena pyriformis*

Compound	Recrystallisation	Specific radioactivity (d.p.m. ^{14}C-mg^{-1})	^{3}H/^{14}C radioactivity ratio	^{3}H/^{14}C atomic ratio
Administered clionasterol (VII)	1	25.630	5.41	
	2	25.234	5.46	
	3	25.051	5.43	1:1
Cholesta-5,7,22-trien-3β-ol (VIII)	1	668	5.35	0.98:1
	2	507	5.47	1.01:1
	3	493	5.39	0.99:1
Methyl isovalerate (IX)		0.62	4.82	0.89:1
Methyl isovalerate after base equilibration		0.68	0.20	0.04:1

during dealkylation in the insect, *Tenebrio molitor*, suggesting that the mechanisms of dealkylation in these two species may be similar. However, fucosterol, which is a probable intermediate during dealkylation in *Tenebrio*, apparently cannot be dealkylated by Tetrahymena [6], although it and isofucosterol can be dehydrogenated. This may reflect the inability of fucosterol to reach the cellular site of dealkylation. On the other hand it may not be involved in dealkylation and thus the pathway may be fundamentally different from that in insects. Even if this is so our results suggest that the dealkylation mechanisms in *Tetrahymena* and insects have at least one common step.

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