Mechanism of Tetrahymanol Biosynthesis: the Origin of the Oxygen Atom

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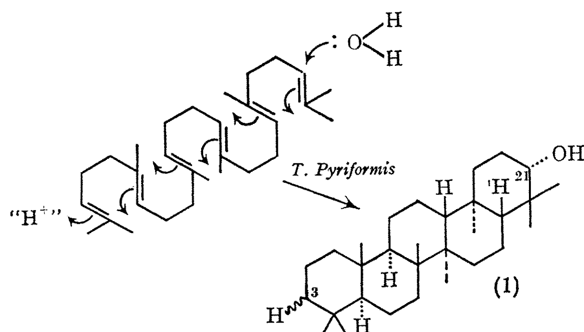
THE biosynthesis[1] of the triterpene tetrahymanol (1) by the protozoan *Tetrahymena pyriformis,* in contrast to the steroids and many other C-3 oxygenated polycyclic triterpenes,[2] does not proceed *via* the intermediacy of squalene 2,3-epoxide.



Instead, a direct proton-initiated cyclisation of squalene takes place.[1,3] The overall process is equivalent to the addition of the elements of water.

Support for this mechanistic scheme is derived from the observation that the *in vitro* biosynthesis of (1) from squalene in D_2O leads to the incorporation of a deuterium atom into tetrahymanol, probably at C-3.[3] Furthermore, tetrahymanol biosynthesis takes place under anaerobic conditions.[4] This suggests that, unlike the oxygen atom of 2,3-oxidosqualene[2a,b] and of lanosterol,[5] the oxygen atom of tetrahymanol is not derived from atmospheric oxygen. We have therefore focussed our attention on the question of the origin of the hydroxy-group of tetrahymanol and now report that we have prepared an active, cell-free, water-soluble enzyme powder from *T. pyriformis,* which when incubated anaerobically with squalene in the presence of $H_2{}^{18}O$ leads to the incorporation of oxygen-18 into tetrahymanol. In the biosynthesis from squalene of the tetracyclic and pentacyclic polyisoprenoids[6] this finding is without precedent.

The protozoa from a 15 l. culture[4] were harvested in a continuous-flow centrifuge at 4°. The packed cells (*ca.* 18 g. wet wt.) were re-suspended in 65 ml. of 15% solution (w/v) of potassium deoxycholate and shaken for 1 min. with glass beads in a Braun Model MSK Cell Homogenizer.

After 20 min., the deoxycholate was precipitated by the addition of a slight excess of 0·77 M-$CaCl_2$ solution. The cell debris and calcium deoxycholate were sedimented by centrifugation (25,000 *g* for 25 min.). The resulting supernatant was dialysed for 7 hr. against distilled water at 0° and then for 1 hr. against 0·1 M-potassium phosphate buffer, pH 7·2. Finally, the dialysate was gently extracted three times with peroxide-free ether and the aqueous enzyme solution was freeze-dried.

A portion of the resulting powder (650 mg.) was dissolved in [^{18}O]water (3 g.; 62·4% excess of ^{18}O) containing 300 μg. of [^{14}C]squalene (1×10^5 d.p.m.) solubilised with 15 mg. of Triton X-100. The mixture was incubated for 15 hr. at 28° in a nitrogen atmosphere and the incubation was terminated by freezing. The water was removed by sublimation, and the residue was saponified[4] with aqueous ethanolic NaOH and extracted with hexane. The hexane extract was chromatographed on a silica gel plate, developed with 10% ethyl acetate–hexane. The tetrahymanol band was re-chromatographed in 5% acetone–methylene chloride. The tetrahymanol thus obtained contained 12% of the ^{14}C-radioactivity of the precursor, indicating that about 36 μg. of [^{14}C]squalene had been converted into tetrahymanol.

The mass spectrum† of the tetrahymanol in the region of the molecular ion indicated the presence of 30·5% of an $M + 2$ species. The fragments‡ at *m/e* 207 and 413 ($M-CH_3$) also showed a 30% enrichment at *m/e* 209 and 415, respectively; on the other hand the peak at *m/e* 412 [$(M + 2)-H_2O$] was unchanged, and hence all the isotopic excess is located in the hydroxy-group.

We conclude that the oxygen atom of tetrahymanol originates from the water of the medium.

Though our results fully support the concept of a proton-initiated squalene cyclization mechanism, they do not distinguish between this and attack of a hydroxide ion at C-21. However, the idea of an anionic (OH^-) attack on squalene would constitute a radical departure from the fundamental concepts of Ruzicka *et al.*,[7] and Cornforth[8] which are based on cationic reactions and rearrangements.

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† A Varian M-66 spectrometer was used, with an ionisation current of 50 μA at 70 ev and a probe temperature of 200°. We thank Mr. D. Quarton for these determinations.
‡ The assignment of this fragment is discussed in ref. 3.

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