Incorporation of Deuterium from Deuterium Oxide into Tetrahymanol biosynthesised from Squalene

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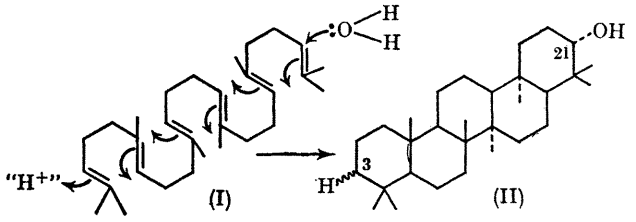
WE have recently demonstrated, both *in vivo*[1] and *in vitro*,[2] that the biosynthesis of tetrahymanol (II)[3] by the protozoan *Tetrahymena pyriformis* does not proceed *via* the intermediacy of squalene 2,3-oxide.[4] This finding was rationalized in terms of a non-oxidative, proton-initiated cyclization[3b,5] of squalene (I).



SCHEME 1

Implicit in this mechanism is the assumption that the cyclization involves an enzyme-mediated acquisition of a hydrogen atom at C-3. It was considered probable that this proton is ultimately derived from the water of the medium. We have therefore carried out the *in vitro* biosynthesis of tetrahymanol in D_2O and have observed the incorporation of deuterium into tetrahymanol.

A 15 l. culture[2] of *T. pyriformis* was harvested after a growth period of 50 hr. by continuous-flow centrifugation at 6600 × g. The resulting pellet of packed cells was rinsed with D_2O and one quarter of it suspended in D_2O (27 ml.) at 0°. The cells were disrupted by shaking with glass beads (10 g. 0·17—0·18 mm. diam.) for 1 min. in a Braun Model MSK Cell Homogenizer with adiabatic cooling by liquid CO_2. The homogenate was decanted and incubated at room temperature with [^{14}C]squalene (5 mg., 4×10^5 d.p.m.) suspended in D_2O (1 ml.) with Triton X-100 (30 mg.). After 18 hr. the incubation was terminated by the addition of ethanol–diethyl ether [3 : 1 (v/v: 300 ml.], the extract was filtered, and the solvents were removed by evaporation under reduced pressure. The aqueous residue was saturated with NaCl and extracted with hexane (3 × 100 ml.). Preparative t.l.c.[2] of this extract, followed by rechromatography of the tetrahymanol band, gave [^{14}C]tetrahymanol (3·98 mg., $5{\cdot}6 \times 10^4$ d.p.m.) which, after recrystallization (EtOH), had a specific activity of $2{\cdot}5 \times 10^4$ d.p.m./mg. Comparison of this specific activity with that of the squalene substrate indicated that 33% of the tetrahymanol was derived from exogenous squalene.

The mass spectrum† of the tetrahymanol in the region of the molecular ion showed it to contain 15% of monodeuteriated species. This value was unchanged after oxidation to tetrahymanone‡ (Table). In neither case were any multiply deuteriated species observed.

TABLE

	Exp. 1		Exp. 2
	Tetrahymanol	Tetrahymanone	Tetrahymanol
Proportion of tetrahymanol formed from [^{14}C]squalene (%)	33		15
Deuterium content (%$^2H_1$)*			
Molecular ion	15	15	10
Fragment (V)	13	13	10
Fragment (IV)†	0	0	0

* Only [$^2H_0$]- and [$^2H_1$]-species were present. All values are the mean of three determinations.

† For the ketone this fragment occurs at *m/e* 205.

The fragmentation[6] of tetrahymanol under electron impact gives two characteristic ions at *m/e* 191 and 207. These are presumed to arise (Scheme 2) from scission of the C(8)–C(14) bond to give (III); subsequent cleavage at 'a' with concomitant hydrogen transfer from ring D leads to ion (IV). A similar sequence involving cleavage at 'b' and hydrogen migration from ring B gives ion (V).§ For the biosynthetic [^{2}H]tetrahymanol, fragments (IV) and (V)

† Mass spectra were measured on both a Consolidated Electro Dynamic Co. Model CE 21-104 and on a Hitachi Model MRU 6D. Identical values for sample deuterium content were obtained from each machine.

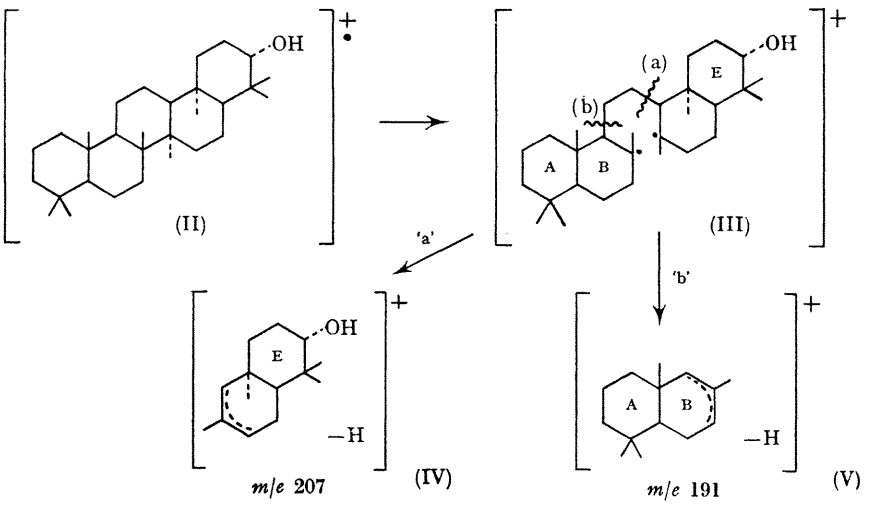
‡ The discrepancy between the percentage of tetrahymanol derived from [^{14}C]squalene and the percentage deuterium found can be ascribed to the isotopic dilution of the D_2O with endogenous water.

§ The assignment of these peaks is supported by the observation that 21β-[^{2}H]tetrahymanol (obtained by reduction of tetrahymanone with $LiBD_4$) shows, in addition to a new molecular ion peak at *m/e* 429 (100% [^{2}H]), a shift of the peak at *m/e* 207 to *m/e* 208 (100% [^{2}H]). The *m/e* 191 fragment is unchanged.

were found to contain 0% and 13% respectively of [^{2}H]-species. An identical distribution of deuterium was observed in the corresponding fragments of tetrahymanone (*m/e* 191, 13% [^{2}H]; *m/e* 205, 0% [^{2}H]). Hence, within experimental error, all of the incorporated deuterium is located in rings A or B, in accord with the postulated biosynthetic mechanism (Scheme 1). The absence of deuterium in the ion (IV) and the agreement between the deuterium content of the parent ion and that of ion (V) exclude the possibility that the deuteriation of tetrahymanol arises from labelling of its endogenous precursors.[7]

proton-initiated squalene cyclization mechanism, they do not distinguish between this and an anionic attack of a hydroxyl at C-21. However, the idea of an anionic (OH^-) attack on squalene would constitute a radical departure from the fundamental concepts of Ruziczka *et al.* which are based on a cationic attack.[8]

Added in proof: We have now improved the cell-free enzyme preparation and obtained a substantially higher incorporation of deuterium into tetrahymanol (37% D_1). This enzyme preparation was also used for the studies of the origin of the hydroxy-group. When the incubation



SCHEME 2

The results described above were confirmed in a separate experiment, for which 15% of the isolated tetrahymanol was found to be derived from exogenous squalene. The deuterium incorporation was 10%, all of which was again located in the ion (V) (Table).

Though our results fully support the concept of a

was carried out in $H_2{}^{18}O$ (62·4% excess ^{18}O), the tetrahymanol contained 30·5% excess ^{18}O.

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