Communications to the Editor

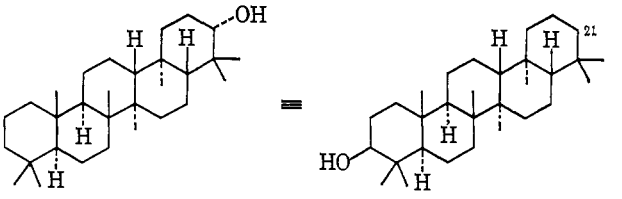
Evidence for a Nonoxidative Cyclization of Squalene in the Biosynthesis of Tetrahymanol

Sir:

The intermediacy of squalene 2,3-oxide in the enzymatic conversion of squalene to lanosterol and cholesterol in the rat-liver system has been elegantly demonstrated.[1-3] Further investigations have shown this epoxide to be efficiently incorporated by a homogenate of *Pisum sativum* into β-amyrin[4] and by the mold *Fusidium coccineum* into fusidic acid.[5] Squalene 2,3-oxide has also been implicated as an intermediate in the biosynthesis of phytosterols by its isolation, along with cycloartenol, from tobacco tissue cultures[6] and by its conversion to cycloartenol in bean leaves.[7]

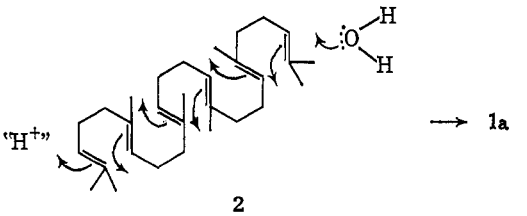
These findings are strongly suggestive of a general role in nature for squalene 2,3-oxide as the precursor of polycyclic triterpenes possessing a 3β-hydroxyl group. However, suggestions have also been made that nonoxidative mechanisms[8] for the enzymatic cyclization of squalene also need to be considered, particularly in the formation of triterpenes lacking a C-3 oxygen function.[9-12] For example, in the cases of zeorin,[13] ambrein,[14] diplopterol,[15] dustanin,[10] and serratene,[12] biosynthetic mechanisms have been postulated involving the cyclization of squalene initiated by direct protonation at C-3. The apparent absence of any direct experimental evidence for the existence of a biosynthetic pathway from squalene to a polycyclic triterpene *not* involving squalene 2,3-oxide as an intermediate led us to carry out the experiments we report here.

For the triterpene tetrahymanol,[16] isolated from the protozoan *Tetrahymena pyriformis*[17] and shown to have structure **1**,[15,17] both the oxidative and the nonoxidative mechanisms appear potentially possible. Thus, tetrahymanol (*cf.* **1b**) might conceivably be derived from cyclization of squalene 2,3-oxide, initiated by protonation on oxygen and terminated either by the direct acquisition at C-21 of hydride (possibly from NADPH or NADH) or by nucleophilic hydroxylation at C-21 to give a 3β,21α-diol with subsequent reductive elimination



1a **1b**

of one of these two equivalent hydroxyl groups. Alternatively, the biosynthesis of tetrahymanol (*cf.* **1a**) might proceed as previously proposed[15] by the equivalent of a proton-initiated cyclization of squalene with terminal nucleophilic hydroxylation, as indicated formally in **2**.



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We have been able to differentiate between these two cyclization schemes by incubating a mixture of ^{14}C-labeled squalene and ^{3}H-labeled squalene 2,3-oxide with *Tetrahymena pyriformis* and analyzing the isolated radioactive tetrahymanol.

[1,5,9,16,20,24-^{14}C]Squalene was obtained from the anaerobic incubation[18] of DL-[2-^{14}C]mevalonic acid dibenzylethylamine salt with the microsomal and supernatant fractions of a rat-liver homogenate.[19] [4,8,12,13,17,21-^{3}H]Squalene was prepared similarly from DL-[5-$^{3}H_2$]mevalonic acid dibenzylethylamine salt and then converted to the 2,3-oxide.[1-3] Both the ^{14}C-labeled squalene and the ^{3}H-labeled squalene 2,3-oxide were purified chromatographically, and the identity and homogeneity of each were established by cochromatography with nonradioactive material and conversion to several derivatives all having the same molar specific activity.

The ^{14}C-labeled squalene (2.3×10^7 dpm; diluted with 2 mg of nonradioactive squalene) and the ^{3}H-labeled squalene 2,3-oxide (3.3×10^8 dpm; diluted with 3 mg of nonradioactive squalene 2,3-oxide) were mixed,[20] and the $^{3}H:^{14}C$ ratio was found to be 14.2. The mixture was dissolved in 12 ml of ethanol, and 1-ml portions were injected into 12 flasks, each containing 500 ml of peptone culture fluid.[21] After a growth

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(16) Gammaceran-3β-ol.
(17) F. B. Mallory, J. T. Gordon, and R. L. Conner, *J. Am. Chem. Soc.*, 85, 1362 (1963).
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(19) N. L. R. Bucher and K. McGarrahan, *J. Biol. Chem.*, 222, 1 (1956).
(20) The viability of the epoxide in this mixture was established by incubation with rat-liver homogenate and isolation of labeled cholesterol with a $^{3}H:^{14}C$ ratio of 11.7.
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period of 40 hr the cells were harvested[22] and lyophilized, the lipids were extracted (1.4 g), and the nonsaponifiable fraction (60 mg) was chromatographed on a silicic acid column (10 g) with 18 successive 25-ml portions of 4% ether in 40–60° petroleum ether. The appropriate fractions were combined to give 16.8 mg of tetrahymanol. After three recrystallizations from 95% ethanol 8.9 mg of pure material was obtained, having a $^{3}H:^{14}C$ ratio of 0.025 (^{14}C specific activity 2.64×10^5 dpm/mg, representing 10% of the total ^{14}C incubated). This corresponds to a gross ^{14}C enrichment factor of 570, so that at least 99.8% of the tetrahymanol originates from squalene directly and is not derived from squalene 2,3-oxide.

Certain other chromatographic fractions of the nonsaponifiable lipids were found to contain large amounts of tritium. From one of these there was isolated, in impure form, material with the properties of squalene 2,3-oxide (cochromatography, derivative formation, and conversion to [^{3}H]cholesterol by a rat-liver homogenate[18,19]). This implies that squalene 2,3-oxide is transported into the cells and that a small portion survives the drastic work-up procedures.

These results are consistent[23] with the hypothesis that the biosynthesis of tetrahymanol involves a nonoxidative, proton-initiated cyclization of squalene.

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(23) The possibility that exogenous squalene 2,3-oxide is not in equilibrium with any endogenous pool cannot be completely discounted. However, precedent[1-6,7] suggests that this is unlikely.

(24) Alfred P. Sloan Research Fellow, 1964–1968.

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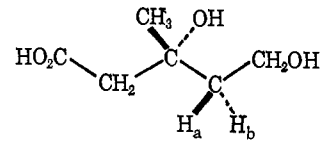
The Biosynthesis of Tetrahymanol from (4*R*)-[4-^{3}H-2-^{14}C]Mevalonic Acid

Sir:

Cornforth, *et al.*, have proved that the biosynthesis of squalene,[1,2] lanosterol,[2] and cholesterol[2] in rat liver preparations entails the stereospecific elimination of the pro-4*S* protons and retention of the pro-4*R* protons of (3*R*)-mevalonic acid (MVA) (**1**). A similar pattern has been observed to hold for the formation of squalene



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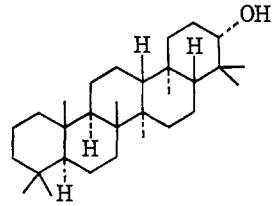
H_a, pro-4*R*
H_b, pro-4*S*

and 3-oxygenated polycyclic triterpenes in other species.[3-5] In contrast, the biosynthesis of rubber[6] and (in part) betulaprenols[7] involves the reverse stereospecificity, with the pro-4*R* protons being eliminated.

The cyclization of squalene[1-5] to 3-oxygenated triterpenes and sterols has been demonstrated to proceed *via* the intermediacy of squalene 2,3-oxide in several species.[8-13] However, we have recently reported[14] evidence for the existence of a new, nonoxidative, "*proton-initiated*" *mechanism of squalene cyclization in* the biosynthesis of the 3-deoxytriterpene tetrahymanol (**2**)[15-17] in the protozoan *Tetrahymena pyriformis*.



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Thus, when this protozoan was grown in a medium containing ^{14}C-labeled squalene and ^{3}H-labeled squalene 2,3-oxide, the resulting tetrahymanol contained only ^{14}C and was devoid of tritium. It therefore became of importance to determine the pattern of proton retention and elimination from C-4 of mevalonic acid that operates in the biosynthesis of triterpenes in *T. pyriformis*. Our examination of this question forms the subject of this communication.

Racemic (3*R*,4*R*-3*S*,4*S*)-[4-^{3}H]mevalonic acid dibenzylethylamine salt (98 μCi), prepared according to the procedure of Cornforth and Popjak,[1] was combined with (3*RS*)-[2-^{14}C]mevalonic acid dibenzylethylamine salt (20 μCi) ($^{3}H:^{14}C$ ratio 4.88, measured on the N-

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(15) Tetrahymanol (**2**) is gammaceran-3β-ol. The symmetry of **2** is such that the hydroxyl group can be considered to be either 3β or 21α. In view of the mechanism of its formation[14] we choose to regard **2** as a 21α-hydroxytriterpene in the present discussion.

(16) F. B. Mallory, J. T. Gordon, and R. L. Conner, *J. Am. Chem. Soc.*, **85**, 1362 (1963).

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