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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(22) R. L. Conner, S. G. Cline, M. J. Koroly, and B. Hamilton, *J. Protozool.*, **13**, 377 (1966).

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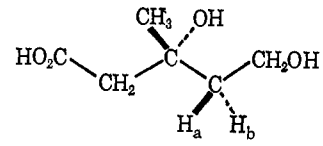
The Biosynthesis of Tetrahymanol from (4*R*)-[4-^{3}H-2-^{14}C]Mevalonic Acid

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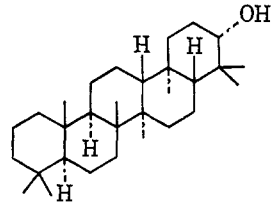
H_a, pro-4*R*
H_b, pro-4*S*

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*.



2

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-

(1) J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popjak, *Proc. Roy. Soc.* (London), **B163**, 492 (1966).

(2) J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popjak, Y. Shimizu, S. Ichii, E. Forchielli, and E. Caspi, *J. Am. Chem. Soc.*, 87, 3224 (1965).

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(5) L. J. Goad and T. W. Goodwin, *ibid.*, **96**, 79P (1965).

(6) B. L. Archer, D. Barnard, E. G. Cockbain, J. W. Cornforth, R. H. Cornforth, and G. Popjak, *Proc. Roy. Soc.* (London), **B163**, 519 (1966).

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(10) J. D. Willett, K. B. Sharpless, K. E. Lord, E. E. van Tamelen, and R. B. Clayton, *J. Biol. Chem.*, **242**, 4182 (1967)

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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.

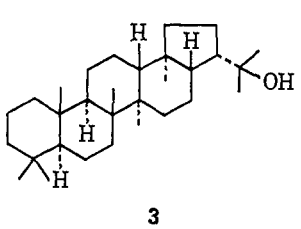
(16) F. B. Mallory, J. T. Gordon, and R. L. Conner, *J. Am. Chem. Soc.*, **85**, 1362 (1963).

(17) Y. Tsuda, A. Morimoto, T. Sano, Y. Inubushi, F. B. Mallory, and J. T. Gordon, *Tetrahedron Letters*, 1427 (1965).

diphenylmethylamide derivative).[18] The resulting mixture was dissolved in 10 ml of water, and 5-ml portions were added to two flasks each containing 500 ml of peptone culture fluid[19] inoculated with *T. pyriformis*. After a growth period of 40 hr, the cells were harvested[20] and subjected to the previously described isolation procedure[14] to give 6.8 mg of tetrahymanol. Sublimation of this material under reduced pressure gave 3 mg of pure tetrahymanol having a $^3H:^{14}C$ ratio of 4.79 (^3H specific activity *ca.* 10^5 dpm/mg, representing 0.7% incorporation); within experimental error, this ratio is equal to that of the starting mevalonic acid. This result, together with the assumption that only (3*R*)-mevalonic acid (**1**) is utilized by *T. pyriformis*, leads to the conclusion that the over-all biosynthetic transformation of **1** into tetrahymanol involves the retention of the pro-4*R* protons. Thus, the pathway from **1** to tetrahymanol in this protozoan is stereochemically analogous with regard to C-4 of mevalonic acid to the pathway followed in the biosynthesis of 3-oxygenated triterpenes in other species.

This conclusion is further buttressed by the isolation of a small amount of a by-product believed to be diplopterol (**3**) having a $^3H:^{14}C$ ratio of 4.86. The identity of **3** is indicated by chromatographic comparisons[21] with an authentic sample[22] and by its mass spectrum: *m/e* 428 (M^+), 413 ($M - CH_3$), 410 ($M - H_2O$), 395 ($M - CH_3 - H_2O$), 370 ($M - CH_3COCH_3$), 369 ($M - CH_3COHCH_3$).

The "proton-initiated" cyclization of squalene[23] to tetrahymanol should lead, barring rearrangements, to the labeling pattern shown in **4** (^3H = T, ^{14}C = ●). To test this hypothesis we have carried out a partial degradation of the doubly labeled tetrahymanol (*ca.* 4.2×10^5 dpm ^3H, *ca.* 9×10^4 dpm ^{14}C) after dilution with 18 mg of nonradioactive **2**. Jones oxidation[24] gave tetrahymanone (**5**),[16,17] mp 286–287°, ir 1705 cm^{-1},



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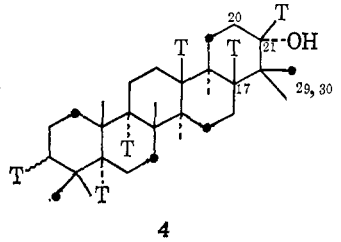

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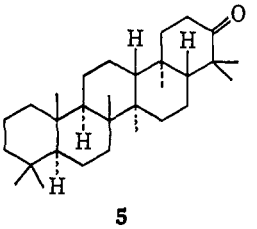
m/e 426 (m^+), with a $^3H:^{14}C$ ratio of 4.12, indicating the loss of one tritium atom from C-21. This ratio was unchanged after treatment of **5** with sodium ethoxide in refluxing ethanol; hence no tritium is present at C-20. Retropinacol rearrangement[25] of tetrahymanol (12 mg) gave hopene-a (**6**),[26] mp 171°, nmr δ 1.53 and 1.70 (two vinylic methyls), *m/e* 410 (M^+). The $^3H:^{14}C$ ratio of **6** was 4.11, virtually identical with that found for **5**, again in accord with the loss of tritium from C-21. Ozonolysis of **6** (zinc–acetic acid work-up) gave the ketone **7**,[26] mp 161–164°, ir 1750 cm^{-1}, *m/e* 384 (M^+). The $^3H:^{14}C$ ratio for **7** was 5.08, corresponding to the loss of one ^{14}C atom, presumably located at C-29 or C-30. Base-catalyzed equilibration



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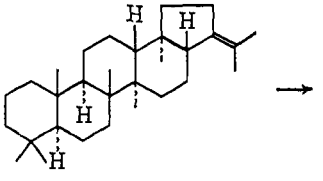

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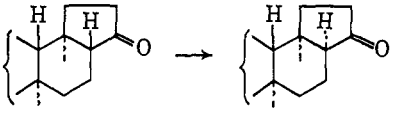

7 **8**

of **7** with ethanolic sodium ethoxide gave the *cis* isomer **8**,[26] ir 1735 cm^{-1}, with the loss of a further tritium atom indicated by the $^3H:^{14}C$ ratio of 3.97; since no label is present at C-20, this tritium atom must be located at C-17. The results are summarized in Table I and are consistent with the anticipated mode of cyclization of squalene.

Table I

Compd	$^3H/^{14}C$ ratio (dpm)	$^3H/^{14}C$ ratio (atomic): Exptl	Theor
1	4.88		
2	4.79	5.89:6	6:6
3	4.86	5.98:6	6:6
5	4.12	5.07:6	5:6
6	4.11	5.05:6	5:6
7	5.08	5.21:5	5:5
8	3.97	4.07:5	4:5
Squalene (from rat liver)	4.81	5.91:6	6:6

Acknowledgments. The work at the Worcester Foundation was supported by the American Cancer Society (P-500 G), the National Science Foundation (GB 5832), and the National Institutes of Health (CA K3 16614-06). The work at Bryn Mawr College was supported by the National Science Foundation (GB

(18) The (3*S*)-mevalonic acids of the racemates are assumed to be biologically inactive.[1] The viability and radiochemical identity of the 3*R* isomers in the mixture was established by incubation with a rat liver homogenate and isolation of labeled squalene with a $^3H:^{14}C$ ratio of 4.81.

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(20) R. L. Conner, S. G. Cline, M. J. Koroly, and B. Hamilton, *ibid.*, **13**, 377 (1966).

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(25) *Cf.* J.-F. Biellmann and G. Ourisson, *Bull. Soc. Chim. France*, 331 (1962).

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4605) and by the college's Fund for the Coordination of the Sciences.

(27) Alfred P. Sloan Research fellow, 1964–1968.

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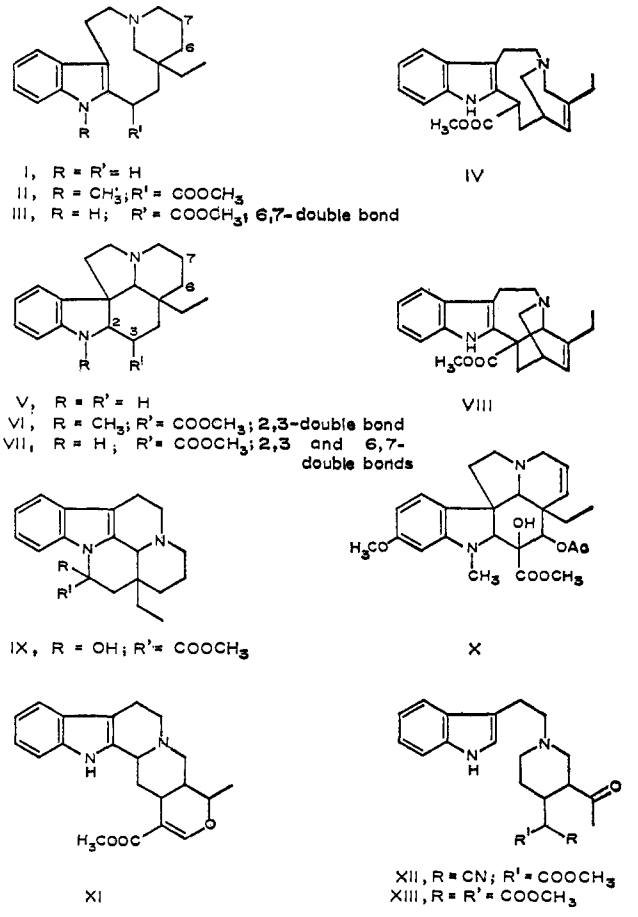
Studies on Indole Alkaloid Biosynthesis

Sir:

In recent years the biosynthesis of indole alkaloids has stimulated considerable interest in various laboratories.[1] Almost without exception these investigations have concentrated on the nature of the "non-tryptophan" unit necessary in the biosynthesis, and numerous elegant experiments are now in hand which establish the monoterpene loganin as playing an important role in this regard.[2–5] Our own interests in this area have been concerned with the later stages of the biosynthetic pathway, *i.e.*, the steps involved *after* the tryptophan–C_{10} "complex" has been formed. Such questions as (a) the structure of this "complex(es)" and (b) the pathways which it follows to elaborate the various families in the indole and dihydroindole series were of prime consideration. This communication presents some of our results in this area.

Of the various postulates which were available, the one proposed by Wenkert[6] was of particular interest in our initial considerations, since it relates directly to our synthetic work in this area. The transannular cyclization reaction developed in our laboratories provides a general entry into Aspidosperma, Iboga, and Vinca alkaloids.[7–10] The fundamental similarity of this latter process to the later steps in Wenkert's postulates provides the stimulus for its evaluation as a biosynthetically significant reaction.

For this purpose, the appropriate nine-membered ring intermediates represented by quebrachamine (I), vincadine (I, R = H; R′ = $COOCH_3$), and vincaminoreine (II) were evaluated as possible precursors of the Aspidosperma and Vinca alkaloids, while the corresponding carbomethoxycleavamine derivative (IV) was studied for its possible role in the biosynthesis of the Iboga family. Numerous experiments were conducted



in *Vinca rosea* Linn and *Vinca minor* Linn plants, and a brief resumé of the results is presented in Tables I and II.

The experimental method associated with the incorporation of large molecular weight compounds in terms of permeability, etc., was appreciated, and the initial experiments dealt with an evaluation of various techniques for the incorporation of such compounds. Table I illustrates the results from the various methods of feeding. It soon became apparent that no particular technique showed any obvious advantage over the others. The most frustrating aspect of these results was our inability to delineate what might be construed as a "positive" demonstration of the transannular cyclization process from the rather trivial oxygen-catalyzed conversion of the intermediates to the alkaloids during the period of incorporation.[11]

In an attempt to obtain internally consistent data which may shed more light on the cyclization reaction, we then turned our attention to a series of experiments in which identical conditions were maintained throughout the entire series. For this purpose, 6-month-old *V. rosea* L. plants were selected, and the incorporation of the appropriate precursor was administered by the cotton wick technique into the stems of the plant. In each instance, the number of plants fed was sufficient to provide the direct isolation of the alkaloids which, without any further dilution with "cold" material, could be crystallized to constant activity. Conversion of each of these into the corresponding hydrochloride

(1) For a recent survey, see A. R. Battersby, *Pure Appl. Chem.*, **14**, 117 (1967).
(2 A. R. Battersby, R. S. Kapil, and R. Southgate, *Chem. Commun.*, 131 (1968).
(3) A. R. Battersby, R. S. Kapil, J. A. Martin, and L. Mo, *ibid.*, 133 (1968), and references cited therein.
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(6) E. Wenkert, *J. Am. Chem. Soc.*, **84**, 98 (1962).
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(10) J. P. Kutney, W. J. Cretney, P. Le Quesne, B. McKague, and E. Piers, *ibid.*, **88**, 4756 (1966).

(11) The conversion of these compounds to the appropriate alkaloids by oxygen in the presence of a metal catalyst was already demonstrated in our laboratory. It was hoped that a much higher level of incorporation in the plants relative to the blank experiment could be obtained.