nonhydrocarbon products occurred at about three-fourths the rate found for squalene under the same conditions. Chromatographic analysis using the thin layer technique (tlc) on silica gel revealed that there was very little product in the R_f range expected for sterols or monohydroxy C_{30} molecules and that the major products exhibited higher R_f values, as expected for substances considerably less polar than sterols. In fact, the chromatographic behavior of the principal product corresponded closely to that of 2,3-oxidosqualene,[6] which suggested that it might be a terminal epoxidation product of 10,11-dihydrosqualene. This was shown to be the case by synthesis of a mixture of 2,3-oxido- and 22,23-oxido-10,11-dihydrosqualene using the bromohydrin method[6,7] starting with 10,11-dihydrosqualene,[8] and comparison with the biologically produced material. The biological and synthetic samples were identical chromatographically using tlc, with different solvent systems on silica gel and silica gel impregnated with silver nitrate, and vapor phase chromatography. Perchloric acid catalyzed hydration of these samples gave products which behaved identically in tlc and exactly as expected for C_{30} vicinal glycol.[6] Finally, the mass spectra of the biological and synthetic oxides were identical and left no doubt that each sample consisted of approximately equal amounts of the two possible terminal epoxidation products.

A second product, of significantly greater polarity than the terminal oxides of I, was present in the biologically produced mixture from 10,11-dihydrosqualene. This was isolated by preparative layer chromatography and analyzed by mass spectrometry, which allowed identification as *2,3:22,23-dioxido-10,11-dihydrosqualene*.[9]

These results, coupled with the finding that 2,3-oxidosqualene is a precursor of sterols in the rat liver system,[6] suggest that the enzyme responsible for the terminal epoxidation of squalene functions almost as well to catalyze epoxidation at either terminal double bond of 10,11-dihydrosqualene. Further, it is evident that the cyclizing enzyme which converts 2,3-oxidosqualene to a steroidal structure rejects both of the terminal oxides from 10,11-dihydrosqualene and allows these to accumulate when 10,11-dihydrosqualene is employed as a substrate in the rat liver system. Thus, some evidence has been provided for the high degree of specificity expected for the steroid-forming enzyme on the basis of the concerted cyclization scheme with a rigidly held, folded[1] substrate.

Lastly, it should be mentioned that 10,11-dihydrosqualene diminishes the rate of conversion of squalene to sterols with rat liver homogenate. However, a comparable effect was observed with squalane and even *n*-eicosane, which indicates that the apparent competitive inhibition is quite nonspecific with regard to structure.

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(7) E. E. van Tamelen and T. J. Curphey, *Tetrahedron Letters*, 121 (1962).
(8) The epoxide obtained from 10,11-dihydrosqualene *via* the monobromohydrin (purified chromatographically) could not be resolved chromatographically. However, it was readily apparent from the mass spectrum that it was a mixture of the two possible *terminal* monooxides, 2,3-oxido- and 22,23-oxido-10,11-dihydrosqualene, in approximately equal amounts.
(9) The ratio of monooxide to dioxide from 10,11-dihydrosqualene under the biological conditions used was *ca.* 5.

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Enzymic Conversion of Squalene 2,3-Oxide to Lanosterol and Cholesterol

Sir:

The role of the C_{30} triterpenoid hydrocarbon, squalene (I), as an intermediate in the biosynthesis of cholesterol and of the naturally occurring tetracyclic and pentacyclic triterpenes is well supported by experimental evidence.[1]

On a theoretical level, past contributors[2,3] considered the biological formation of the polycyclic triterpenoids from squalene by a mechanism in which cyclization was initiated by attack of OH^+ on one of the terminal double bonds of the hydrocarbon chain and proceeded in a concerted fashion without the formation of any stable intermediates. The structure of the product, according to these proposals, would be determined by the conformation imposed upon the squalene chain by the enzyme catalyzing the cyclization. The rearrangements of methyl groups and hydrogen atoms of squalene which were presumed to occur during its cyclization to lanosterol were rationalized on the basis of conformational stereoelectronic considerations. Several features of these proposals have been tested by experiment.

The results of Tchen and Bloch[4] supported the concerted mechanism for this conversion, and convincing evidence for the postulated shifts of methyl groups and hydride ions has also been obtained.[5-7] The precise role of oxygen in the cyclization process has not been investigated, however, although it has been demonstrated by Tchen and Bloch[8] that atmospheric oxygen provides the 3β-hydroxyl group of lanosterol and that NADPH is required in the reaction. While these observations are consistent with cyclization mechanism A, they do not exclude other possibilities. One of these is mechanism B in which oxygen first adds to the terminal double bond of squalene to yield squalene 2,3-oxide which then undergoes a proton-initiated cyclization.

It recently became possible to test mechanism B when a procedure was developed in one of our laboratories[9]

(1) R. B. Clayton, *Quart. Rev.* (London), **19**, 168 (1965).
(2) (a) L. Ruzicka, *Experientia*, **9**, 357, 362 (1953); (b) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chem. Acta*, **38**. 1890 (1955).
(3) G. Stork and A. Burgstahler, *J. Am. Chem. Soc.*, 77, 5068 (1955),
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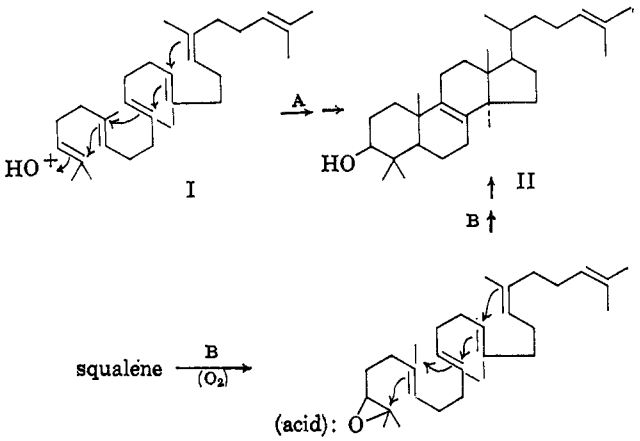
(7) J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popjak, Y. Shimizu, S. Ichii, E. Forchielli, and E. Caspi, *J. Am. Chem. Soc.*, **87**, 3224 (1965).
(8) T. T. Tchen and K. Bloch, *J. Biol. Chem.*, **226**, 921 (1957).
(9) E. van Tamelen and T. J. Curphey, *Tetrahedron Letters*, **3**, 121 (1962).

for the selective epoxidation of a terminal double bond in a polyenoid structure such as squalene. The potential of such polyene epoxides for both enzymic and nonenzymic cyclization is of obvious interest, and we wish briefly to report the results of biological experiments which strongly suggest squalene 2,3-epoxide as a normally occurring intermediate in the enzymic cyclization of squalene.

Squalene labeled with ^{14}C was prepared by anaerobic incubation of mevalonic acid-2-^{14}C with a liver homogenate prepared according to Bucher and McGarrahan,[10] saponification of the incubation mixture, and isolation of the hydrocarbon fraction by chromatography of the nonsaponifiable extract on alumina. The highly labeled squalene-containing fraction was further purified by thin layer chromatography (tlc) on silica gel G in hexane, diluted with nonradioactive



material to a suitable specific activity, and purified further *via* the thiourea adduct. The recovered material was then converted to the 2,3-epoxide by the method of van Tamelen, *et al.*[9,11] The epoxide (R_f 0.44) was purified by tlc on silica gel G with 5% ethyl acetate in hexane. This system gives a complete separation of the epoxide from any unchanged squalene (R_f 0.85).

The epoxide with a specific activity of 3800 dpm/μg was incubated for 2 hr with the liver enzyme system of Bucher and McGarrahan at a concentration of 100 μg (added in propylene glycol)/3 ml of homogenate, in an atmosphere of either oxygen or of nitrogen. The products of the incubations were isolated by saponification, extraction of the nonsaponifiable fractions with hexane, and separation by tlc on silica gel G in 25% ethyl acetate in hexane. Cholesterol (R_f 0.28) and lanosterol (R_f 0.44) were identified by comparison with the pure compounds as markers visualized by exposure to iodine vapor. From incubations under N_2 10–20%[12] of the recovered activity traveled with the R_f of lanosterol and less than 1%[12] appeared in the cholesterol region. The remainder was recovered as apparently unchanged epoxide. The material recovered from the lanosterol band was converted to the trimethylsilyl ethers and subjected to gas and liquid chromatography (glc) on a column of thermostabilized polydiethylene glycol succinate (5% on Chromosorb P) at 195°, N_2 flow rate 210 cc/min. The material emerged as two peaks with retention time, relative to cholestane, 3.7 (lanosterol) and 2.7 (dihydrolanosterol). The materials were recovered and assayed for ^{14}C. The lanosterol peak contained 30% of the recovered activity and 60% appeared in the dihydrolanosterol peak. A result consistent with this distribution was obtained when a sample of the radioactive material recovered from tlc was combined with nonisotopic lanosterol, recrystallized four times, acetylated, recrystallized, and converted to the 3β-acetoxy 24,25-dibromide (mp 167–169°) as described by Johnston, Gautschi, and Bloch.[13]

The specific activity of the material (calculated as lanosterol) fell progressively through these operations from 500 to 160 dpm/mg; the activity remained stable after two crystallizations of the acetate dibromide.

From incubations carried out in oxygen approximately 15%[12] of the recovered activity was found in the cholesterol region on tlc, only about 3%[12] in the lanosterol region, and the remainder in the squalene epoxide region. The major product was identified as cholesterol by admixture with nonisotopic cholesterol, crystallization, acetylation, precipitation as the acetate dibromide, regeneration of the sterol acetate by zinc–acetic acid treatment, and final recrystallization. Through all of these operations the specific activity remained essentially unchanged (200–200 dpm/mg). Further samples of the recovered radioactive sterol, without dilution, were converted to the methyl ether and subjected to glc on polydiethylene succinate as described by Clayton.[14]

The emergent peak (retention time, relative to cholestane, 3.8, identical with that of pure cholesterol) contained all of the recovered activity and, when collected fractionally across the peak, no change in specific activity was detected.

To demonstrate the biosynthesis of the epoxide, isotopically labeled squalene was incubated in the presence of 100 μg of nonisotopic squalene 2,3-oxide/3 ml of liver homogenate. When the nonsaponifiable material recovered from these incubations was separated by tlc on silica gel G in 5% ethyl acetate–hexane, the epoxide band contained approximately 5% of the activity as a discrete peak.

These results are all consistent with the conversion of squalene to lanosterol *via* the epoxide (mechanism B). Moreover, extensive studies[15] of the nonenzymic cyclization of squalene epoxide have shown that its proposed biological reactivity compares and contrasts significantly with the nonenzymic reactivity; in the latter case the steric specificity imposed by the enzyme is obviously lacking.

Acknowledgment. The authors wish to express their thanks to Dr. Marjorie Lindberg and Miss Betty Swyrd for assistance with some aspects of the enzyme

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(11) The nmr spectrum and elemental analysis of the unlabeled epoxide support the assigned structure, also confirmed in that the nature of a further transformation product, the terminal bisepoxide, had been unequivocally established.[9]

(12) Assuming utilization of only one enantiomer, these yields should be doubled.

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Magnetic Resonance Studies of Ion Solvation. The Coordination Number of Gallium(III) Ions in Aqueous Solutions

Sir:

An extensive ^{17}O nuclear magnetic resonance study of aqueous solutions containing Ga^{3+} ions has been carried out. Herein are reported the results of the coordination number determinations.[1]

Two independent methods were used to determine the number of water molecules in the sphere of hydration of the Ga^{3+} ions. The coordination number was determined from the ratio of areas of the bound to the free water, where one is shifted relative to the other by the addition of Co^{2+} ions, and also was determined from a measurement of the shift of the free water relative to reference pure water.[2-4]

To pure water containing about 10% ^{17}O and acidified with perchloric acid four successive amounts of cobaltous perchlorate were added. The paramagnetic shift of the ^{17}O of the water molecules was measured relative to a pure water reference. The following results obtained are given in Table I. Extrapolation of these results to 1 mole of Co^{2+}/55.5 moles of H_2O gives 11000 cps, corresponding to 1905 ppm.

Table I[a]

Detmn	n_{H_2O}	n_{HClO_4}	$n_{Co(ClO_4)_2}$	$\Delta f_{ref/sol}$, cps
1	59.64	0.95	0.0598	600
2	60.13	0.95	0.1303	1320
3	60.59	0.95	0.1973	1972
4	60.96	0.95	0.2498	2615

[a] Amounts expressed in millimoles; the four points were on a straight line.

To solution 4, 0.5805 g of $Ga(ClO_4)_3 \cdot (9.38 \pm 0.23)$-$H_2O$ was added. The measured paramagnetic shift for this solution was 2370 cps, whereas the calculated is 2142 cps. Assuming that the increase in the observed shift relative to the calculated is caused by the Ga^{3+} ions retaining part of the water in the hydration sphere, the coordination number is calculated to be 6.28 ± 0.26.

For the same solution the ratio of the areas of the absorption signals of the bound to the free water yielded a coordination number of 5.89 ± 0.20 (as an average of four signals).

The two methods are seen to be in good agreement. They are, however, sensitive to different factors. The method, based on the measurement of the shift itself, requires accurate determination of the water of hydration of the salt. As a matter of fact the error in the coordination number of the ion in the solution will be the error in the determination of the hyration of the salt. On the other hand, the method based on the ratio of the areas of the free to the bound water signals will not require this. The advantage of the method based on the measurement of the shift is that it is easier to measure the shift of the water in bulk than to measure areas. In particular the measurement of the area of the bound water to the desired precision—accurately enough to determine the hydration numbers—is very elaborate. Depending on the circumstances one of the two methods should be preferred. In the case of the Ga^{3+} ion the experimental error of the two methods came out to be about the same.

As given above, the paramangetic shift of the free water relative to the pure water was found to be 2370 cps. The shift of the free water relative to the bound was 2190 ± 10 cps. Hence there is a paramagnetic shift of 180 cps of the bound water relative to the pure.

The width of the bound water signal at 35° was 753 cps. The width of the free water signal was 580 cps.

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(4) M. Alei and J. A. Jackson, *ibid.*, **41**, 3402 (1964).
(5) Work performed at the Lawrence Radiation Laboratory, University of California, Berkeley, Calif.

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Complete Sequence of Biosynthesis from *p*-Hydroxybenzoic Acid to Ubiquinone[1]

Sir:

A complete biosynthetic sequence can now be formulated for the pathway from *p*-hydroxybenzoic acid (I) to ubiquinone (IX). Since *p*-hydroxybenzoic acid is a precursor for ubiquinone in various microorganisms[2] and in animals,[2-4] this sequence may be generic for many species of life utilizing ubiquinone in electron transfer of respiration and coupled phosphorylation. However, the diversity of life utilizing ubiquinone may signify that pathways alternative to this sequence will be found.

Four new quinones, apparent precursors of ubiquinone, have been isolated by extensive fractionation of a lipid extract from *Rhodospirillum rubrum*. Structural studies show these products to be 2-decaprenyl-6-methoxy-3-methyl-1,4-benzoquinone (VII, $n = 10$),

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