

The Biological Synthesis of Cholesterol

Konrad Bloch

In the early 1930's after decades of effort the structural elucidation of cholesterol had reached the stage of completion, and with this achievement one of the most brilliant chapters of organic chemistry came to a close. To chemists and biochemists alike the structure of cholesterol at once presented a biosynthetic problem of exceptional challenge. At first sight the molecular architecture of cholesterol seemed enigmatic and devoid of any clues as to how this complex molecule might be constructed from the smaller molecules available in the cell. It is, therefore, all the more remarkable that much of the early speculative thinking came so close to predicting some of the general principles that were eventually shown to operate in sterol biosynthesis. The early proposals were mainly concerned with the problem of ring formation, and quite uniformly they envisioned an origin of the tetracyclic steroidal ring system from an appropriately folded long-chain precursor. This intuition proved to be correct. All of the speculative schemes have in some way influenced the research that was to take place later, but none equalled in perspicacity L. Ruzicka's unifying hypothesis on the common origin of terpenes and steroids and the suggestion by Sir Robert Robinson that cholesterol might arise by cyclization of the hydrocarbon squalene. These bold and appealing ideas carried perhaps more weight because they had some experimental evidence to support them. Already in 1926 Shannon, following a proposal by

Heilbron, Kamm, and Owens (1), had shown that squalene fed to animals increases the cholesterol content of the tissues (2).

The phase of continuous research on cholesterol biosynthesis begins in 1937 with two independent and remarkably complementary investigations. In the course of their pioneering studies on intermediary metabolism with the aid of stable isotopes, Rittenberg and Schoenheimer at Columbia arrived at the conclusion that the process of cholesterol formation involves the coupling of smaller molecules, "possibly those which have been postulated to be intermediates in the fat and carbohydrate metabolism" (3). During the same year Sonderhoff and Thomas, noting an incorporation of trideuterioacetate into the unsaponifiable materials of yeast, stated: "Es lässt sich darauf schliessen dass die Sterine der Hefe auf einem recht unmittelbaren Wege aus der Essigsäure entstehen" (4).

As a graduate student under Hans T. Clarke at Columbia and later as an assistant of R. Schoenheimer, I was introduced to the new and powerful tool of isotopic tracers and shared the intellectual excitement of applying it to problems of biosynthesis, a previously closed area of metabolic investigation. Schoenheimer, a pathologist and biochemist, had long been interested in the origin and metabolism of cholesterol and was planning to pursue this problem actively when his brilliant career came to an untimely end in 1941. Taking up the promising leads which already existed, Rittenberg and I began a systematic study of the utilization of labeled acetic acid for cholesterol synthesis in animal tissues and could soon show that acetate contributes in a major way to the synthesis of both the aliphatic side chain and the tetracyclic moiety of the sterol molecule (5, 6). After my move to

the University of Chicago these studies were continued with the objective of establishing the origin of all carbon atoms of the cholesterol skeleton and with the hope that the pattern of distribution would be informative. Individual positions of the iso-octyl side chain and the angular methyl groups were relatively accessible to chemical degradation and analysis, and with information available for only a few carbon atoms we ventured the prediction that a two-carbon metabolite of acetate is the principal if not the sole building block of cholesterol (7) (Fig. 1). The arguments were largely deductive, and therefore more convincing evidence for the exclusive origin of the sterol molecule from acetate was sought. A mutant of *Neurospora crassa*, which E. L. Tatum had isolated, served this purpose admirably because a deficiency in pyruvate metabolism made the growth of the mutant dependent on exogenous acetate. Cells of the mutant strain grown on labeled acetate produced ergosterol essentially without dilution of the isotope, and this proved that no other carbon source contributed significantly to the synthesis of the sterol skeleton (8).

One might ask why the biochemical mutant technique which has contributed so decisively to the charting of biosynthetic pathways has not been employed more often in studying sterol biosynthesis. Attempts to use it may have been made, but apparently without success. Sterol-less mutants of molds or fungi have never been reported, and any search for bacterial mutants would have been in vain because the pathways of sterol biogenesis do not exist in the bacterial phylum. Nevertheless, mutant strains of bacteria have in one instance been exceedingly important, if inadvertently, for the discovery of a key sterol precursor.

The thinking in our laboratory about modes for constructing larger molecules from acetate units was greatly influenced by information which had come from studies on rubber biosynthesis. Bonner and Arreguin had demonstrated the utilization of acetate for the biosynthesis of this isoprene polymer, and they speculated how three acetate molecules might combine to form the requisite isoprenoid subunits for the macromolecule by way of acetoacetate and β -methylcrotonic acid (9) (Fig. 2). Assuming that their scheme was also applicable to the construction of cholesterol, we could predict how isotopes from the two car-

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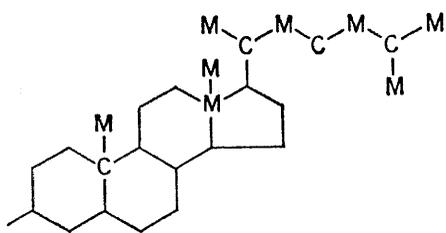
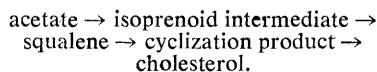


Fig. 1. Origin of some of the carbon atoms of cholesterol from methyl (*M*) and carboxyl (*C*) carbon atoms of acetic acid. [Wuersch, Huang, and Bloch (10)]

bons of acetate should be arranged in certain portions of the molecule. For the isooctyl side chain of cholesterol the distribution pattern could be checked experimentally (10). Prediction and experimental fact agreed satisfactorily, and therefore the view evolved quite naturally that cholesterol, like many other natural substances, was derived from a polyisoprenoid intermediate (Fig. 3). For this view to take hold, the ground was, of course, well prepared by Robinson's hypothesis, according to which cholesterol was formed by the cyclization of squalene, a polyisoprenoid hydrocarbon (11, 12). At this stage an outline of the major stages of the overall process emerged:



In order to verify the general hypothesis I attempted to demonstrate the formation of squalene from labeled acetate in the shark, an animal species which, for reasons yet unknown, accumulates this hydrocarbon in unusually large amounts. The project involved an excursion into comparative biochem-

istry, for which the Biological Station in Bermuda was chosen. Experimentation with intact sharks or with the lipid-rich shark liver posed, however, considerable technical difficulties and I failed in the desired objective. Fortunately, the more realistic decision to study squalene synthesis in the liver of the rat succeeded in the laboratory at Chicago in the skillful hands of R. G. Langdon (13). Once available in labeled form, squalene could readily be shown to serve as a precursor of cholesterol in the intact animal (14). Only much later (in 1958) was the squalene-sterol conversion rigorously proved with authentic, chemically synthesized ^{13}C -all-*trans*-squalene (15).

Cyclization of Squalene

The available information thus left little doubt about the key role of squalene in sterol biosynthesis, but whether the cyclization proceeded by folding of the hydrocarbon chain, as Robinson had specified, could not yet be answered. It was obviously necessary to know whether the arrangement of acetate carbons in the steroid skeleton conformed with prediction, as it did in the sterol side chain. This involved the formidable chemical task of a complete carbon-by-carbon dissection of the cholesterol nucleus, eventually achieved by the elegant and definitive experiments of Cornforth and Popják between 1953 and 1957 (16) (Fig. 4). In the meantime the Robinson hypothesis needed to be examined more closely for different reasons. Lanosterol, the sterol from wool fat, had been known for some time to combine in its structure features of both the sterols and the pentacyclic triterpenes, but it was not until 1952 that Ruzicka, Jeger, and their collaborators furnished unequivocal proof of the 4,4,14-trimethylcholestane structure of lanosterol (17). I learned of this development in the fall of 1952 after a lecture I presented to the chemistry department at Harvard. In a discussion that followed—joined by Professor E. R. H. Jones (Sir Ewart)—Professor R. B. Woodward argued incisively for a novel type of squalene folding (Fig. 5). The appeal of his proposal was that it uniquely rationalized the structure of lanosterol. It also placed lanosterol in the pathway between squalene and cholesterol, at least by inference. One major and immediately verifiable consequence of the mechanism suggested by Woodward was that it predicted an alternate arrangement of acetate carbons in the steroid molecule differing from the pattern called for by Robinson's scheme in four of the 27 positions (at C_7 , C_8 , C_{12} , and C_{13}) (Fig. 6). Returning to the Chicago laboratory, I was able to confirm the origin of C_{13} from a methyl group of acetate in accordance with prediction. This welcome result encouraged us to commit ourselves to the new and much more plausible scheme for the cyclization of squalene (18). A similar proposal was made independently by Dauben *et al.* shortly thereafter (19). The "correct" origin of C_7 , another of the critical carbon atoms, was demonstrated later (20, 21), and the evidence became com-

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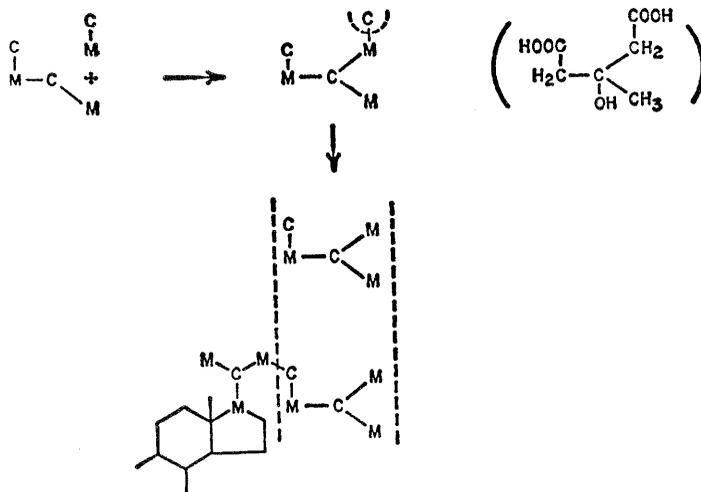
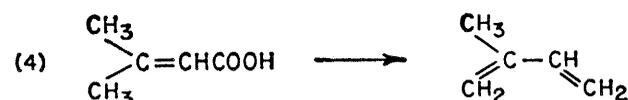
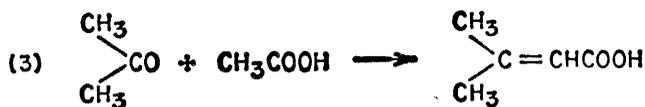


Fig. 2 (left). Proposed reactions for the formation of isoprene from acetic acid. [Bonner and Arreguin (9)] Fig. 3 (right). Predicted and experimental distribution of acetate carbon atoms in cholesterol formed from isoprenoid precursors. At upper right, β -hydroxy- β -methylglutaric acid (see text).

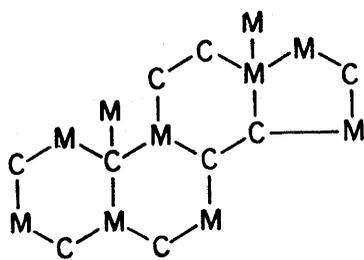


Fig. 4. Distribution of methyl (*M*) and carboxyl (*C*) carbon atoms of acetic acid in the nucleus of cholesterol. [Cornforth and Popják (see 16)]

plete when Cornforth and Popják identified C_8 and C_{12} in the course of their comprehensive degradation of the cholesterol nucleus. The same investigators greatly strengthened the as yet loose and speculative fabric by showing that squalene derived from methyl-labeled acetate was indeed isotopically constituted as the hypothesis had predicted (22). The essential demonstration of the conversion of squalene to lanosterol (23) and of lanosterol to cholesterol came in due course (24). In these experiments we followed the directions of N. L. R. Bucher for preparing liver homogenates (25), a technique which proved invaluable in all subsequent studies on sterol biogenesis.

In 1953, Ruzicka and his colleagues published the first of two classical papers on the origin of terpenes and sterols and on the biogenetic relationships which the various structures suggested. Numerous natural products, some clearly related, others related in

a much less obvious way, were now connected by plausible mechanisms and revealed as stemming from the main trunk or from the branches of a single biogenetic tree (26). The views of the Zürich school were developed further in 1955 in a publication (27) which Cornforth (28) has aptly referred to as "the apotheosis of the isoprene rule." The stereochemical arguments developed here led, *inter alia*, to a greatly refined formulation for the mechanism by which squalene cyclizes to lanosterol (Fig. 7). Three of their main postulates were of special interest to us: (i) cyclization is initiated by a formal cation OH^+ attacking the position of squalene, which becomes C_3 of the sterol nucleus; (ii) cyclization results from a series of interactions of electrophilic centers with electrons of suitably disposed double bonds, affording lanosterol without stabilization of products at any intermediary stage; and (iii) once the tetracyclic ring system is established, two hydrogen atoms and two methyl groups migrate to neighboring positions in a concerted rearrangement that terminates in lanosterol. Submitting these postulates to experimental test we obtained results which were in each instance in accordance with theory. T. T. Tchen found that molecular oxygen, the formal equivalent of OH^+ , and not water, is the source of the 3-hydroxyl group of cholesterol. Furthermore, his experiments showed that the enzymatic cyclization of squalene to lanosterol in a D_2O medium proceeds without

attachment of deuterium to carbon, as it should if any (but the last) in the series of presumptive carbonium ions fail to stabilize (29).

Methyl Migration

Another predictable feature of the squalene-lanosterol transformation was the relocation of two branched methyl groups in the squalene skeleton. This could occur by a single 1,3 methyl shift, or more likely by the migration of two methyl groups to their adjacent carbon atoms. The problem was resolved in favor of two 1,2 methyl shifts (from C_8 to C_{14} and from C_{14} to C_{13}) by Cornforth and Popják and their associates (30), and independently by us (15). The two groups followed separate approaches, but both exploited the fact that mass spectroscopy enables one to distinguish the masses of molecules differing by one or more isotopic atoms. In our laboratory the problem of methyl migration was studied with synthetic all-*trans*-squalene (31) suitably labeled by ^{13}C . This was transformed enzymatically into lanosterol and the product was exhaustively oxidized to acetic acid, which in turn was converted to ethylene for mass spectrographic analysis. Briefly, the results demonstrated that the methyl group at C_{14} of lanosterol was not the same group that had originally been attached to the corresponding position of squalene but that it had come there from a neighboring

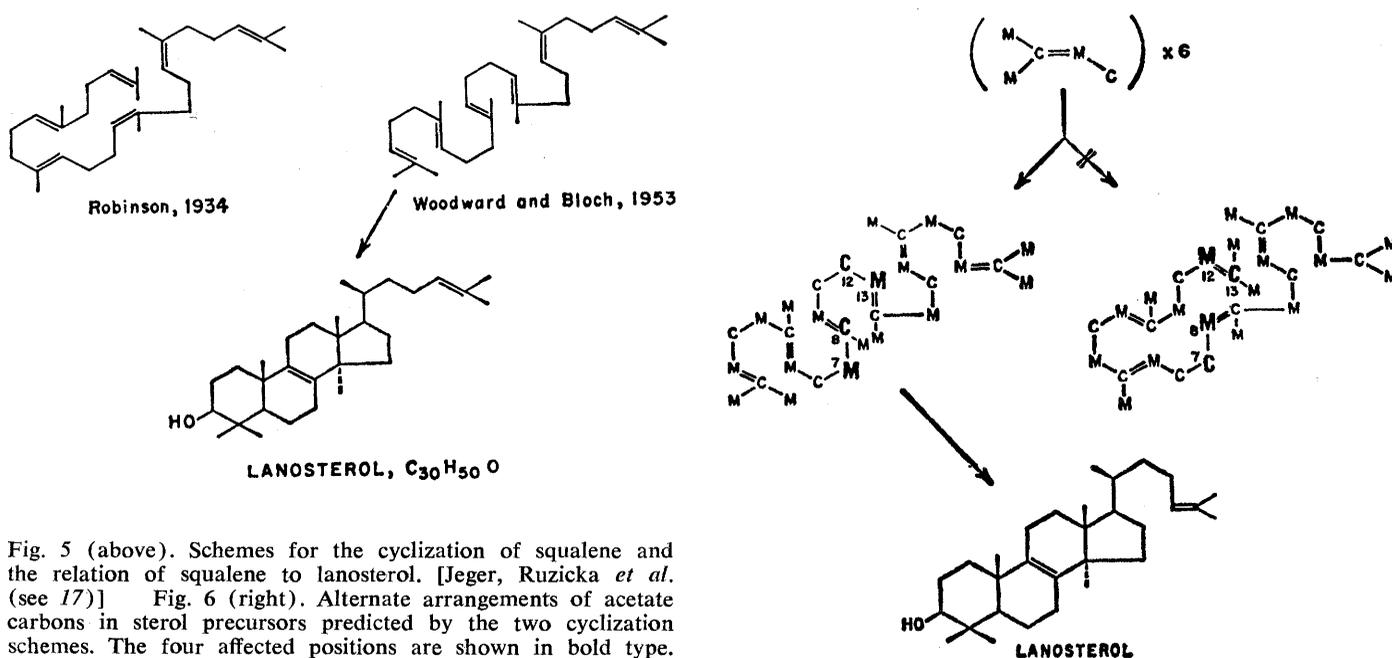


Fig. 5 (above). Schemes for the cyclization of squalene and the relation of squalene to lanosterol. [Jeger, Ruzicka *et al.* (see 17)] Fig. 6 (right). Alternate arrangements of acetate carbons in sterol precursors predicted by the two cyclization schemes. The four affected positions are shown in bold type.

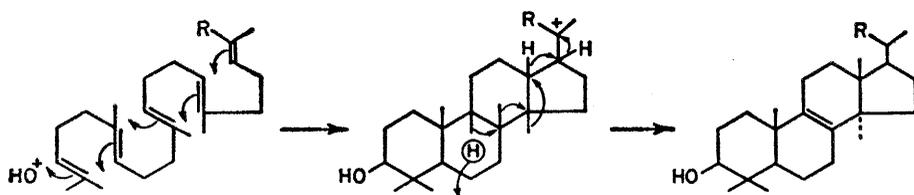


Fig. 7. Mechanism for the cyclization of squalene to lanosterol (see 26, 27).

position by a 1,2 methyl shift. It logically followed that the second of the shifting methyl groups also migrates to an adjacent position. The English workers used for the same purpose ^{13}C -mevalonic acid, which they converted to cholesterol. Again the acetic acid obtained by oxidation was analyzed, the results permitting the additional conclusion that the methyl migration from C_{14} to C_{13} is intra- and not intermolecular. The successful outcome of the studies on squalene cyclization has been particularly gratifying. It demonstrates exceptionally well that the organic chemist and the biochemist must interact closely if they wish to solve the problems of biochemical reaction mechanisms.

Mevalonic Acid

Continuing a historical rather than a systematic description of cholesterol biosynthesis and returning to the earlier stages of the process, we proceed to the discovery of mevalonic acid, the substance that proved to be the link between acetic acid and the biological isoprene unit. Since 1952 several laboratories had searched for branched-chain C_5 or C_6 intermediates without discovering any compounds that showed the requisite biological activity. β -Hydroxy- β -methylglutaric acid, a substance known at that time to occur in plants (32), seemed structurally attractive as a condensation product of 3 moles of acetate and as a precursor of a branched C_5 unit (33), but its biological activity was disappointing. (Later, it was recognized that hydroxymethylglutarate is in fact an intermediate, but only in the form of the monocoenzyme-A derivative.) The breakthrough came in 1956 with the isolation of mevalonic acid by Wright, Folkers, and associates at the Merck Sharp & Dohme laboratories (34). The original purpose of these investigations was to isolate and characterize a factor which was exceptionally active as a substitute for acetate in the nutrition of acetate-requiring strains of *Lacto-*

bacillus acidophilus (35). Noting the structural resemblance of mevalonic acid and hydroxymethylglutarate—their carbon skeletons are identical (Fig. 8)—Tavormina, Gibbs, and Huff tested the bacterial growth factor and found it to be remarkably active as a precursor of squalene and sterol (36). Conversion was essentially quantitative, assuming that only one of the enantiomorphs was active. The discovery of mevalonic acid as a key intermediate in terpene and sterol biosynthesis is one of the examples of serendipity on which the progress of science depends so critically. The example is the more remarkable because up to the present the function of mevalonic acid in the lactobacilli which require it has remained unknown. Sterols play no essential role in the metabolism of bacteria, nor is mevalonic acid required by lactobacilli as a precursor of polyisoprenoid derivatives.

The enzymatic link between acetyl coenzyme A (CoA) and mevalonate by way of acetoacetyl CoA and hydroxymethylglutaryl CoA was soon established in the laboratories of Rudney (37, 38) and of Lynen (39, 40) (Fig. 9). Clearly, Lynen's fundamental discovery of acetyl CoA in 1951 paved the way for studying the early steps in sterol biosynthesis, as it did for the understanding of all phases of fatty acid metabolism. One important issue that remains to be resolved is the origin of the acetoacetyl CoA that enters into the synthesis of hydroxymethylglutarate. Conceivably the initial condensation to the four-carbon level involves an acetyl unit and a malonyl unit (41), as in the synthesis of long-chain fatty acids. Energetically this mechanism is certainly more favorable than reversal of the β -ketothiolase reaction. We have observed, however, that, in biotin-deficient yeast, sterol synthesis from acetate proceeds normally, whereas fatty acid synthesis is greatly impaired (42). This finding would seem to argue against any role of biotin- CO_2 and therefore of malonyl units in sterol synthesis from acetate, at

least in some organisms. The purposes of metabolic regulation would also be served better if the acetoacetyl precursors for sterols and fatty acids were furnished by independent mechanisms. This would allow for control of the entry of acetyl CoA into the two pathways at the earliest possible stage.

Mevalonic acid has the same oxidation level as isoprene and is formally convertible to isoprene by decarboxylation and by the loss of two molecules of water. The possibility was, therefore, attractive that the subunits condensing to the presumptive mono- and sesquiterpenoid intermediates might be closely related to isoprene itself. In our first experiments, designed to test this view, we studied the retention of tritium in the enzymatic conversion of 5-di- ^3H -mevalonate to squalene. We found that in this transformation only a small fraction of the 12 hydrogen atoms attached to C_5 of the six participating mevalonate molecules was removed (43). Thus one of the two bond-forming centers (C_5) seemed to remain in the reduced state during the process of carbon-carbon bond formation. Later experiments with D_2O and 5- D_2 -mevalonic acid strengthened this conclusion and allowed us to make the same deduction for carbon atom 2 of mevalonate. Lacking still any direct knowledge of the nature and number of intermediates, we were nevertheless in a position to interpret these results in terms of a general reaction mechanism for the coupling of C_5 or C_6 subunits (44) (Fig. 10). Bond formation had to occur without loss or reintroduction of hydrogen at the reacting centers—that is, by interaction of mevalonic acid derivatives containing $-\text{CH}_2-$ groups at both the C_2 and C_5 positions. From the same experiments it could be inferred further that the removal of the tertiary hydroxyl group and the loss of the carboxyl function of mevalonic acid proceed concertedly to a C_5 compound bearing a methylene group. Possible structures for the reactive condensing unit were thereby limited to isoprene itself or a derivative of isopentene (44).

Concurrently we investigated the requirements for cofactors in squalene synthesis from mevalonic acid in yeast extracts and found that both adenosine triphosphate (ATP) and reduced triphosphopyridine nucleotide (TPNH) had to be supplied to the enzyme system (43). Suspecting phosphorylated intermediates, T. T. Tchen isolated a stable monophosphate of mevalonic

acid and the requisite kinase enzyme (45, 46). The derivative was the 5-monophosphate ester, as Lynen was able to show (40). Commenting on the mevalonic kinase reaction, Tchen pointed out that a phosphate ester grouping would be an appropriate leaving anion for hydroxyl elimination and thus aid in forming the anticipated terminal methylene group of the biological isoprene unit. As subsequent developments showed, this guess needed to be modified only slightly.

Sequence from Mevalonic Acid to Squalene

5-Phosphomevalonic acid was an excellent substrate in squalene synthesis, but since it was not converted unless once again ATP was provided for the reaction, additional phosphorylation steps were indicated. At this time (May 1958) investigators in the field met in London at a CIBA Symposium on Terpene and Sterol Biogenesis. It was clear that three laboratories were on the trail of several phosphorylated derivatives of mevalonic acid: Lynen's group and ours working with yeast extracts and the group of Popják and Cornforth with liver preparations. We reported the isolation of two new de-

rivatives of mevalonic acid, one containing two atoms of phosphorus per molecule and cleaved by mild acid hydrolysis to orthophosphate and mevalonic monophosphate, and another yielding orthophosphate and Δ^3 -isopentenol when digested with snake venom phosphatase. Our first and premature structural assignments for these substances were 3,5-diphosphomevalonate and isopentenylmonophosphate (47). These structures were soon revised when additional experiments showed that the five-carbon compound contained two atoms of phosphorus and therefore had to be isopentenylpyrophosphate (48, 49). On the basis of this assignment the logical structure for the six-carbon compound was mevalonic acid-5-pyrophosphate (49) (Fig. 11). Lynen and his associates came to the same conclusion and, moreover, provided firm proof for the identity of isopentenylpyrophosphate by chemical synthesis (50).

Chemically the most interesting of the reactions in the sequence from mevalonic acid to isopentenylpyrophosphate is the ATP-facilitated decarboxylative β -elimination of mevalonic acid-5-pyrophosphate, the step that generates the biological isoprene unit. The concerted nature of this reaction had been anticipated by the results of our

earlier deuterium studies (44). It was, therefore, gratifying to find that purified "anhydrodecarboxylase" catalyzed the coordinated removal of the carboxyl group and of the tertiary hydroxy function as postulated (44). Data obtained with ^{18}O suggest that 3-phosphomevalonic-5-pyrophosphate is a transitory intermediate (51), ATP serving as the phosphorylating agent for the tertiary hydroxyl group and thereby promoting its elimination (Fig. 12).

In the symmetrical squalene molecule there are two terminal isopropylidene groups, and, since the "biological isoprene unit" has the isopropenyl structure, two of the six isopentenyl groups must isomerize at some stage of squalene synthesis. One step that might appropriately initiate the condensation of C_5 units is an interaction of an isopropenyl ester with enzyme in a double displacement reaction to yield pyrophosphate and isopentenyl enzyme, the latter isomerizing subsequently to the dimethylallyl structure (47). This possibility now seems unlikely. Lynen proposed an alternative mechanism (Fig. 13) involving the isomerization of free isopentenylpyrophosphate to dimethylallyl pyrophosphate prior to condensation (50). Support and proof for this mechanism were provided by Lynen and his associates when they iso-

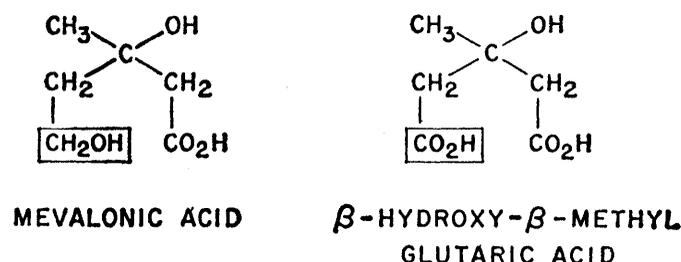


Fig. 8. Structural relation of mevalonic acid and β -hydroxy- β -methylglutaric acid. [Merck Sharp & Dohme Laboratories, 1956]

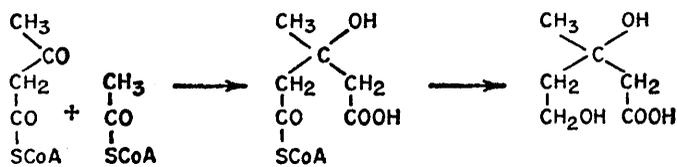


Fig. 9. Biosynthesis of mevalonic acid. [Rudney (37); Lynen, 1958]

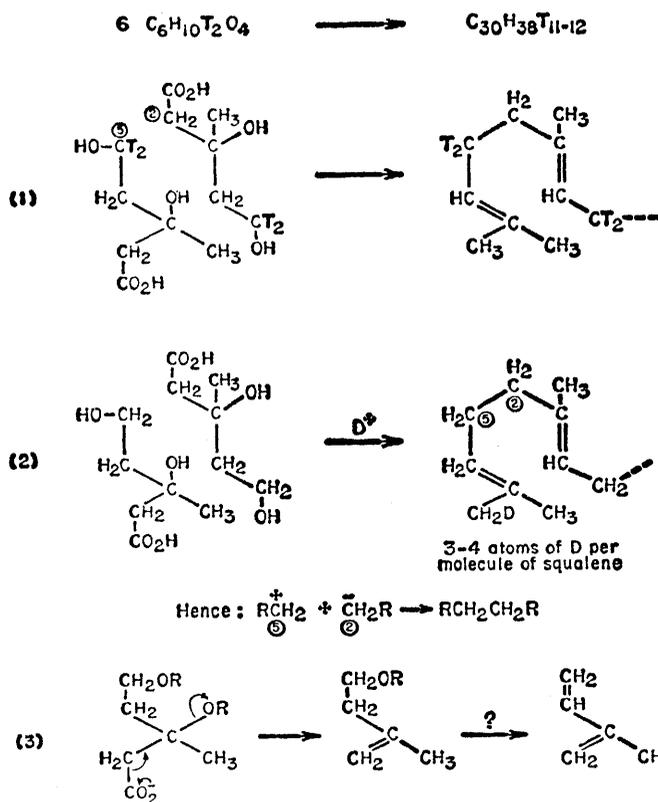


Fig. 10. Studies with heavy hydrogen pertaining to the structure of the biological isoprene unit.

lated the requisite isomerase and showed that this enzyme is an essential component in the coupling system when isopentenylpyrophosphate is the sole substrate (52). The discovery of geranylpyrophosphate (53) and of farnesylpyrophosphate (50) in Lynen's laboratory completed the description of the biosynthetic pathway and of each

enzymatic step up to the sesquiterpene stage (Fig. 14). As Popják was able to show, the same intermediates participate in the synthesis of squalene by liver enzymes (54, 55).

For those participating in the characterization of the intermediates between mevalonic acid and squalene this period was especially exciting. The

chemistry was novel and essentially unexpected, and the means for obtaining structural proof of microgram quantities were highly unorthodox by conventional standards. The nearly total reliance in the crucial experimentation on the tracer technique and its various extensions seems almost without parallel.

The polymerization of C_5 units by the joining of methylene groups has no precedent among enzymatic mechanisms for forming carbon-carbon bonds. One can formulate both the initial interaction of two C_5 units and subsequent C_5 additions as resulting from a nucleophilic attack of the electrons available in the exomethylene group of isopentenylpyrophosphate on an incipient cation formed by pyrophosphate elimination from the allylpyrophosphate. All these events are viewed as being concerted (Fig. 13a). This mechanism is undoubtedly attractive and useful as a working hypothesis, but it lacks the proof which only studies with purified enzymes can provide. Guided by the concepts prevalent in organic chemistry we have adopted, perhaps too readily, the practice of formulating reactions in terpene and sterol biosynthesis as synchronous events. Yet modern enzyme chemistry points to alternative solutions. For example, covalent enzyme-substrate complexes might be formed initially between the allylpyrophosphate and the condensing enzyme with elimination of pyrophosphate (Fig. 13b). Allyl-enzyme rather than the free allylpyrophosphate would then react with a second C_5 unit to form the new carbon-carbon bond.

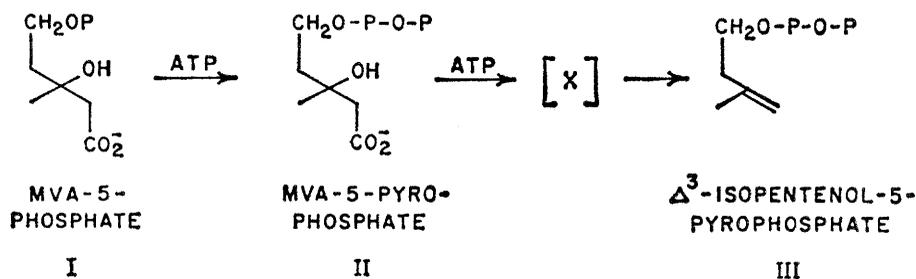


Fig. 11. Phosphorylated derivatives of mevalonic acid. [Structure I, Tchen (45); structure II, Chaykin *et al.* (49) and Henning *et al.*, 1959; structure III, Chaykin *et al.* (49) and Lynen *et al.* (39)]

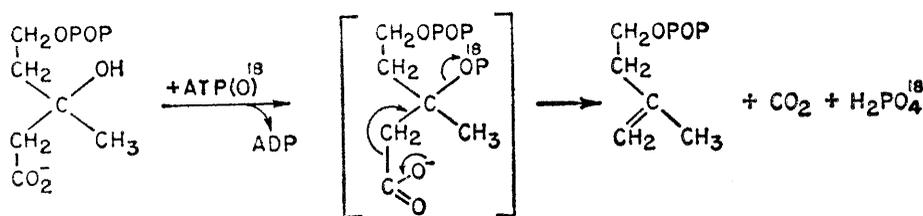


Fig. 12. Decarboxylative β -elimination of mevalonic acid-5-pyrophosphate to isopentenylpyrophosphate. [de Waard, Phillips, and Bloch, 1959; Lindberg *et al.* (51)]

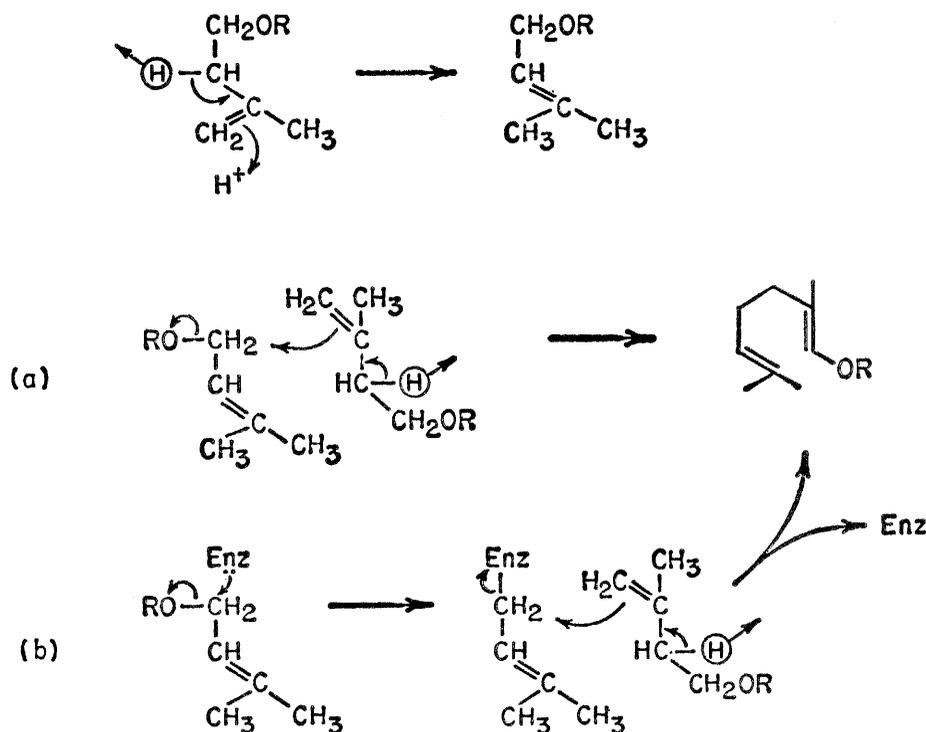


Fig. 13. Isomerization of isopentenylpyrophosphate and mechanisms for the condensation of isopentenyl units. [Lynen *et al.* (53)]

from liver (55) afford as end products predominantly, if not exclusively, farnesylpyrophosphate. Longer chains—for example, C_{20} pyrophosphate—are produced at less than 1/100 the rate observed for the C_{15} pyrophosphate. The second specific enzyme, a purified terpene synthetase isolated in our laboratory from *Micrococcus lysodeikticus*, produces mainly geranylgeranylpyrophosphate, possibly small amounts of the C_{25} homologue, but no C_{15} pyrophosphate (56). The chain-length specificities of the two enzymes reflect in a striking way the economy of cellular processes. Termination of the chain at the C_{15} stage is to be expected when the enzyme functions in a biosynthetic pathway that leads to squalene and to sterol, as it does in yeast and liver. In bacteria, sterol synthesis does not occur, but since *Micrococcus* produces carotenoids it is reasonable to assume that this bacterial terpene synthetase specializes in the formation of geranylgeranylpyrophosphate, the C_{20} precursor of the carotenoids.

In the formal sense, squalene formation can be viewed as resulting from the dimerization of two farnesyl units and, as Lynen has shown, these are provided by farnesylpyrophosphate (50). The process is reductive, and this accounts for the TPNH requirement which we noted in early studies of squalene synthesis from mevalonic acid (43). Again, we are dealing with an interaction of two reactive methylene groups, as in the coupling of isopentenyl units. However, the resemblance is only superficial since the farnesyl dimerization is reductive and involves the union of two like groups (tail to tail). In $C_5 + C_5$ condensations isopentenyl units are joined head-to-tail fashion, and a reducing agent is not needed. Several ingenious schemes have been proposed for the last step in squalene synthesis (57). From the elegant studies of Cornforth and Popják (58) we know the steric course of the coupling reaction, but otherwise the mechanism has remained elusive. Some of our own contributions to this subject have unfortunately obscured rather than clarified the problem. Early experiments on squalene synthesis in D_2O or from D-mevalonate in crude yeast extracts seemed to indicate that the formation of the central carbon-carbon bond in squalene entailed the loss and the reintroduction of two hydrogen atoms at the bond-forming

centers (44). Cornforth and Popják therefore proposed, quite logically, that squalene synthesis occurred by way of dehydrosqualene, an intermediate with a centrally located double bond (57). However, this idea had to be abandoned when the English workers showed by mass spectrometry that only one of the hydrogen atoms linked to the reacting groups is replaced during the C_{15} - C_{15} coupling, and not two (59). Our later results fully confirmed this finding (60). One important mechanistic detail has thus been clarified, but the mystery surrounding the C_{15} - C_{15} condensation has yet to be solved. At the moment it would appear that the decision will lie between two mechanisms favored by Cornforth and Popják, and assumed to involve either an isomerization of one of the farnesyl units to a nerolidol derivative prior to condensation or, alternatively, a Stevens-type rearrangement of an enzyme-bound farnesyl residue (61). Squalene synthetase,

which may or may not be a single enzyme, has not yet been obtained in soluble form, and this greatly restricts the approaches that can be taken to elucidate the mechanistic details of this interesting reaction. The same technical difficulties must be overcome if we are to learn more about the mechanism of squalene cyclization and about the subsequent steps in the biosynthesis of cholesterol.

Late Stages of the Cholesterol Pathway

The enzymatic characterization of the late stages of the cholesterol pathway has hardly begun, and at present little more is known about this subject than the identities of some of the intermediates. From the nature of the chemical changes involved in the transformation of lanosterol to cholesterol one would nevertheless estimate that they require a large number of enzymes, probably more than half of the total

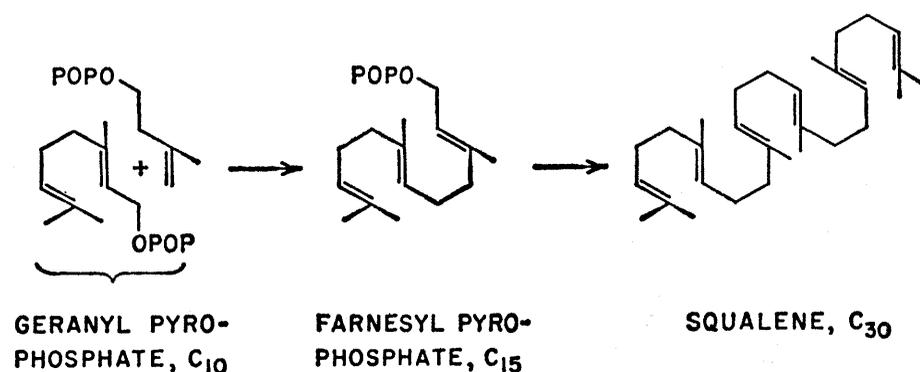


Fig. 14. Mono- and sesquiterpene precursors of squalene. [Lynen *et al.* (50, 53)]

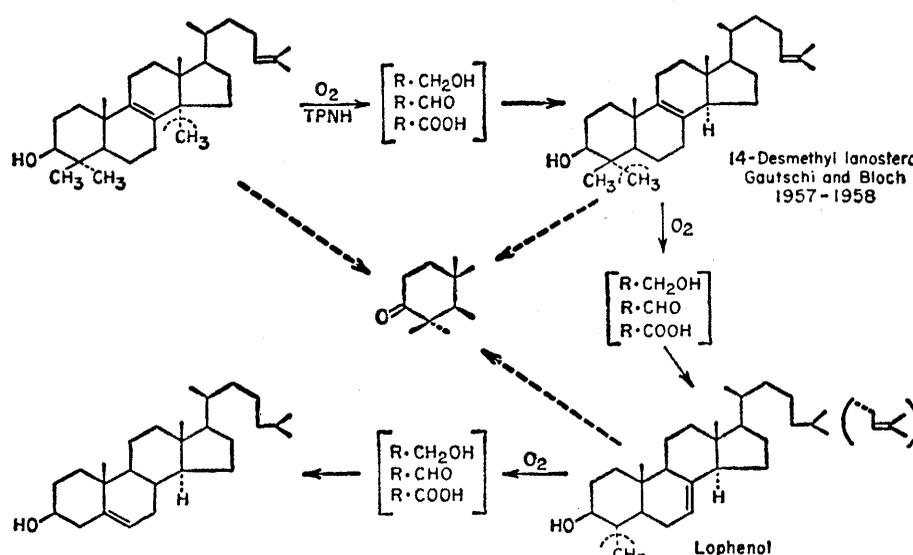


Fig. 15. The oxidative removal of methyl groups from lanosterol and the final stages in the biosynthesis of cholesterol. [For lophenol, see Wells and Niederhiser (64); Djerassi *et al.* (65)]

that comprises the entire pathway (Fig. 15). These steps necessarily include the removal of the methyl group at C₁₄ and those at C₄, the reduction of the isooctenyl side chain, and the (formal) transfer of the double bond from the 8,9 to the 5,6 position in the ring system.

Lacking any clues as to the order of the transformations and expecting the intermediates to occur in traces only, we chose the "pulse" technique of radioactive labeling for detecting new metabolites and the sequence in which they arise. When labeled acetate was injected into rats and the animals were sacrificed a few minutes later, several radioactive materials of unknown identity were obtained on chromatography of the nonsaponifiable tissue fraction (62). One of the fractions, somewhat more polar than lanosterol but less polar than cholesterol, was suspected of being a partially demethylated derivative of lanosterol. This was too small in amount to be weighed or seen, and structural identification of the metabolite had to be attempted without recourse to traditional chemical methodology or physical measurements. Various pieces of information could be obtained by a combination of radiochemical and enzymatic techniques similar in principle to those which led to the identification of isopentenylpyrophosphate. They all showed that the unknown differed from lanosterol only in lacking the methyl substituent at C₁₄. The structure deduced for the metabolite was, therefore, 14-desmethyl lanosterol or 4,4-dimethylcholesten-8,24-diene-3 β -ol (63). Conditions for accumulating this sterol in biological systems have not yet been devised, and only the 24,25-dihydro derivative, not the sterol itself, has been chemically synthesized. The physical properties of 14-desmethyl lanosterol are, therefore, not known. It also remains to be demonstrated that the 14-nor sterol is a metabolite of lanosterol, even though no alternatives are obvious. At any rate, the identification of this short-lived intermediate and its facile conversion to cholesterol disclosed the order of the reactions by which lanosterol is transformed to cholesterol. The view that demethylation at C₁₄ precedes the removal of the methyl groups at C₄ was soon supported by the isolation of two monomethylsterols, 4- α -methyl- Δ^7 -cholesten-3 β -ol (methostenol or lophenol) from animal (64) and plant sources (65), and of 4- α -

methyl- Δ^8 -cholestenol from tumor tissue (66). Both these substances are converted to cholesterol and are obviously metabolites of lanosterol. The sequence of demethylation reactions is not necessarily the same in all systems, judging from structural evidence alone. For example, Djerassi has isolated from plant sources a 14-methylcholestane derivative, 14- α -methyl- Δ^8 -cholesten-3 β ,6 α -diol (Mcdougallin) (67), which may or may not be on the main metabolic path. In animal tissues, however, this sterol is inert (68).

In isolated liver the demethylation of lanosterol proceeds only in the presence of oxygen and then produces 3 moles of CO₂ per mole of cholesterol formed (69). Presumably each of the three methyl groups is initially hydroxylated in an oxygenase-type reaction and then oxidized to aldehydic and carboxylic derivatives. Finally, the C₁ substituent is eliminated from the sterol skeleton by decarboxylation. There is ample structural precedent for the stepwise oxidation of methyl groups in the steroids and in the cyclic terpenes (Soyasapogenol, aldosterone, abietic acid). Also, the oxygen-dependent hydroxylation of terminal methyl groups is now a well-established enzymatic reaction for aliphatic chains. One of the presumptive partially oxidized intermediates, 4-hydroxymethylene- Δ^7 -cholesten-3-one, has been synthesized (70) and it shows the requisite biological activity, producing a C₂₇ sterol as well as CO₂ in the usual liver system. This transformation occurs anaerobically as well as in air, as one would expect if only the initial attack on the methyl group required molecular oxygen. The corresponding carboxylic acid, 4-carboxy- Δ^7 -cholesten-3-one, is also decarboxylated by liver homogenate, but, as the rate of the reaction is also quite rapid in the absence of enzyme, the significance of this reaction as an enzymatic process is still in question (71). The enzymatic removal of the oxidized one-carbon substituents appears, in any event, to be very similar mechanistically to the corresponding nonenzymatic reactions. Decarboxylation is assisted either by a neighboring double bond or by a keto group in the β -position (72).

The remaining structural changes which still have to be placed in the proper order are the elimination of the double bond in the isooctenyl side chain and the formal transfer of the nuclear double bond from position 8,9 to 5,6. Whether the side-chain

reduction occurs necessarily at one specific point and whether this involves early or late intermediates in the lanosterol-cholesterol transformation is not yet clear. The structure of desmosterol ($\Delta^{5,24}$ -cholestadienol) certainly suggests that this reductive step can be the very last one of the sequence (73). By the same token, however, the existence in the tissues of several side-chain saturated sterols (dihydrolanosterol, lophenol, 4- α -methyl- Δ^8 -cholestenol, Δ^7 -cholestenol) argues for reduction at a much earlier stage of lanosterol metabolism. Either the reducing enzyme systems are fairly nonspecific (74) or the substrate specificity is not the same in all the tissues in which sterol synthesis occurs. For very similar reasons we do not yet know whether the double-bond shift from the $\Delta^{8,9}$ to the Δ^7 position occurs early or late during the lanosterol-cholesterol transformation. It is, however, well established, mainly by the work of Frantz and his colleagues, that this isomerization precedes the entry of the 5,6 double bond (75). A $\Delta^{5,7}$ -cholestadienol is undoubtedly on the main reaction path yielding cholesterol in a final reductive step.

Perspective

The direction that further research on terpene and sterol biosynthesis will take and the aspects to be emphasized seem clearly indicated. Biosynthetic studies generally begin at the level of intact animals or whole cells and progress gradually to *in vitro* experimentation. Only with isolated and ultimately pure enzymes can one hope to understand reaction mechanisms, and for many steps of cholesterol biosynthesis this is still a distant goal. We are already quite well informed about the early phase of the sequence which covers the steps from acetyl CoA to farnesylpyrophosphate. This is undoubtedly so because the respective enzymes are soluble and lend themselves to the traditional techniques of protein purification. It is perhaps not without significance that all the substrates for these soluble enzymes are freely water-soluble CoA derivatives or pyrophosphate esters. By contrast, squalene and the subsequent members of the biosynthetic chain are highly hydrophobic molecules, and the enzymes that act upon them are tightly bound to particulate elements of the cell. Con-

ceivably the transformation of these lipid substrates requires more complex catalytic systems in which lipoproteins themselves may play a part. At any rate the available methodology is clearly inadequate for manipulating many of the enzymes that act on lipophilic substrates.

A more highly developed enzymology of the cholesterol pathway is also needed for a rational approach to the problem of metabolic regulation. This is clearly a matter of broad concern, transcending purely academic curiosity. A great variety of environmental, dietary, and hormonal factors have been studied and shown to influence the rate of cholesterol synthesis, but the evidence is still too scanty for specifying the point or points at which physiological control is most effectively exerted. If the principle of negative feedback operates in sterol biosynthesis, as it does in so many pathways, then the first specific step of the sequence should be rate-limiting, and it should be sensitive to cholesterol, the end product of the biosynthetic sequence. With these considerations in mind attention has been focused on the reduction of hydroxymethylglutaryl CoA to mevalonic acid as a likely control site. There is experimental support for this view, particularly from the work of Bucher, who has shown that the profound effects of cholesterol feeding, of x-radiation, and of starvation on the rates of cholesterol synthesis appear indeed to be exerted at a stage close to or immediately before the formation of mevalonic acid (76). This concept of a homeostatic control continues to receive attention (77), and it is to be hoped that the rapid progress that is being made in the elucidation of regulatory processes will lead also to a better understanding of the mechanisms that control cholesterol biosynthesis.

Along with the interest in chemical and enzymatic aspects of cholesterol biosynthesis there has been a growing appreciation of the role which sterols might play as fundamental cell constituents. The known metabolic transformations of cholesterol, the conversions to steroid hormones and bile acids, surely serve a specialized function since they take place only in vertebrate species. However, in many tissues and cells the function of cholesterol is clearly not metabolic. In organisms which do not metabolize sterol but nevertheless produce or require it—and this is true for all but the most primitive

forms of life—sterols must play some role as structural elements of the cell. Comparative biochemistry suggests what this function might be. Sterols have not been found in any bacteria or in the blue-green algae—that is, in primitively organized cells which lack the various membrane-bound intracellular organelles. The elaboration of membrane-enclosed structures devoted to specialized functions is now viewed as a landmark in evolutionary diversification (78), and it would appear that the parallel development of the biosynthetic pathway to sterols is one of the biochemical expressions of these morphological events. The sterol molecule is not distributed at random inside the differentiated cell but appears to be mainly associated with the cytoplasmic membrane and its endoplasmic extensions. We do not yet know why and for what specific purpose the sterol molecule was selected during the evolution of organisms. One may speculate, however, that the rigidity, the planarity, and the hydrophobic nature of the molecule provide a combination of features that is uniquely suitable for strengthening the otherwise fragile membrane of the more highly developed cell.

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Predation, Body Size, and Composition of Plankton

The effect of a marine planktivore on lake plankton illustrates theory of size, competition, and predation.

John Langdon Brooks and Stanley I. Dodson

During an examination of the distribution of the cladoceran *Daphnia* in the lakes of southern New England, it was noted that large *Daphnia*, although present in most of the lakes, could not be found among the plankton of several lakes near the eastern half of the Connecticut coast. The characteristic limnetic calanoid copepods of this region, *Epischura nordenskioldi* and *Diaptomus minutus*, and the cyclopoid *Mesocyclops edax* were also absent. Small zooplankters were abundant, especially the cladoceran *Bosmina longirostris* and the copepods *Cyclops bicuspidatus thomasi* and the small *Tropocyclops prasinus* (1).

All of these lakes lacking large zooplankters have sizable "landlocked" populations of the herring-like *Alosa pseudoharengus* (Wilson) = *Pomolobus pseudoharengus* (Fig. 1), known by several common names including "alewife" and "grayback" (2). This is originally an anadromous marine fish, breeding populations of which have become established in various bodies of fresh water, including Lake Cayuga, New York, and the Great Lakes (3).

The marine populations live in the coastal waters of the western Atlantic, from the Gulf of St. Lawrence to North Carolina, and ascend rivers and streams to spawn in springtime. The young return to the sea in summer and autumn (4). The seven Connecticut lakes (Fig. 2) with self-perpetuating populations of alewives are within about 40 kilometers of the present coastline, and each is drained directly, by a small stream or river, or indirectly, through the estuaries of the larger Connecticut or Thames rivers, into Long Island Sound (Fig. 3). As such streams and rivers are normally ascended by marine alewives, it is assumed that the establishment of these self-sustaining populations in the lakes is natural.

The "alewife lakes" are diverse in area and depth. Although we have not examined the food of the alewives in these Connecticut lakes (alewives are difficult to catch), studies in other lakes have revealed that planktonic copepods and Cladocera are the primary food. The indifference of alewives to non-floating food is not surprising in view of the adaptation of the parent stock to feeding on zooplankton in the open waters of the sea (5).

The dominant crustaceans in the plankton of all the alewife lakes are

the same small-sized species, *Bosmina longirostris* (or *Ceriodaphnia lacustris*) being most numerous; *Cyclops bicuspidatus thomasi* and *Tropocyclops prasinus*, present in varying ratios, are also numerous. By contrast, in the non-alewife lakes *Diaptomus* spp. and *Daphnia* spp. are always dominants, usually accompanied by the larger cyclopoids *Mesocyclops edax* and *Cyclops bicuspidatus thomasi*. The absence of all but one of these last-named larger zooplankters from the lakes inhabited by the planktivorous alewife may be due to differential predation by the alewives. The elimination of these pelagic zooplankters allows the primarily littoral species, such as *Bosmina longirostris*, to spread into the pelagic zone, from which, we conclude, they would otherwise be excluded by their larger competitors.

Changes in Crystal Lake Plankton

An opportunity to test this hypothesis was provided by the introduction into a lake in northern Connecticut of *Alosa aestivalis* (Mitchell), "glut herring," a species closely related, and very similar, to *Alosa pseudoharengus* (6). The plankton of Crystal Lake had been quantitatively sampled by one of us in 1942 before *Alosa* was introduced. At that time the zooplankton was dominated by the large forms (*Daphnia*, *Diaptomus*, *Mesocyclops*) expected in a lake of its size (see Table 1). Resampling 10 years after *Alosa* had become abundant should reveal plankton similar in composition to that common in the lakes with natural populations of *Alosa pseudoharengus* and unlike that characteristic of Crystal Lake before *Alosa* became abundant.

The plankton of the entire water column of Crystal Lake was sampled quantitatively on 30 June 1964 (7). All the crustacean zooplankters caught in the 1942 and 1964 collections (a total of 6623 specimens) were

The authors are affiliated with the Biology Department, Yale University, New Haven, Connecticut, where Dr. Brooks is associate professor of biology.