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ATHEROSCLEROSIS

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Edited by

GREGORY PINCUS

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Shrewsbury, Massachusetts

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PREFACE

Recognizing the extent and importance of current investigations on the role of hormones in atherosclerosis and the need for critical and informed communication between the investigators in this general field, the Endocrinology Study Section of the National Institutes of Health appointed a subcommittee to organize a conference that would bring together the various disciplines involved. This committee consisted of Col. Marshall E. Groover, Dr. Samuel Gurin, Dr. Robert T. Hill, Dr. Leo T. Samuels, Dr. Jeremiah Stamler, Dr. Alfred E. Wilhelmi, and Dr. Gregory Pincus, chairman. Aided by a grant made to the Endocrinology Study Section by the National Heart Institute, the committee organized a meeting which lasted from March 11th to March 14th, 1958. The meeting was held at the Alpine Rose Lodge in Brighton, Utah, with the kind cooperation of Dr. Leo Samuels and his colleagues at the University of Utah Medical School.

The program of the meeting herein published involved the presentation of a series of papers and the recording of the discussions of these papers. This program was designed to cover five major aspects of research pertinent to the problem of hormones and atherosclerosis. First of all, the problem of cholesterol metabolism is discussed in various aspects, including the nature of cholesterol biosynthesis, the hormonal influences thereon, and certain considerations of cholesterol catabolism. Second, the role of hormones in lipogenesis and lipid transport, particularly in relation to atheromatous lesions, is discussed. Third, available data on the influence of various hormones on experimental atherosclerosis are reviewed. Fourth, the much discussed problem of the interrelationship between blood lipids and the endocrine state in animals and man is presented in detail. Finally, we have a series of papers on clinical-biological interrelationships important to the consideration of endocrine influences on human atherosclerosis. With this coverage of a wide range of investigations, it is hoped that a thorough airing has been given to fundamental data and the concepts which have arisen from these data.

Stated in the simplest possible terms, there is abundant evidence that cholesterol biosynthesis, transport, degradation, and excretion may come under hormonal influence. Furthermore, different endocrine systems may have different effects upon these processes. Not all of the endocrine influences are clear cut and vividly definable. Also, the fundamental concept that the nature of cholesterol metabolism in the mammals affects in one way or another the phenomenon of atherosclerosis may be questioned either in detail or in extenso. Nonetheless, hor-

monal influences on the basic processes are definitively indicated, and a discussion of these influences is clearly worth while. Again, it is elementary that one may establish atherosclerotic lesions along with accompanying blood phenomena in experimental animals. In these conditions, hormones may act both prophylactically and therapeutically. How relevant the results with experimental animals are to human atherosclerosis is certainly a matter for discussion.

Experimental data presented here, as well as a host of observations in the literature, demonstrate conclusively that the sex hormones, thyroid hormone, and adrenocortical hormones may definitively affect the level of circulating blood lipids in man. Although a correlation appears to exist between these blood lipid levels and the degree of development of atherosclerosis, a major problem is whether the hormonal effects on lipid levels are also effects on tissue atherosclerosis. The possibility of a disengagement of the factors concerned with blood levels from those concerned with tissue lesion development certainly requires exploration.

The cardinal question for therapy in atherosclerotic disease is the utility of hormones as therapeutic agents. This is certainly discussed in detail in the latter part of this book. However, equally important is the problem of the role of hormones in the etiology of human disease. Attempts at rational therapy thus far have perhaps ignored the pro-atherosclerotic effect of certain endocrine states.

It is the hope of the committee that this presentation of the discussion of the foregoing problems and related matters will be welcomed for purposes of orientation in this complex field. In addition, we feel that a stimulation to further critical inquiry may be one of the fruits of the efforts here incorporated. We believe that you will find in these pages clear evidence of the devoted pursuit of investigation on the part of the participants in the symposium. To these participants, the committee is extremely grateful.

GREGORY PINCUS

Shrewsbury, Massachusetts
November, 1958

CONTENTS

CHAPTER 1

Biosynthesis of Cholesterol

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If the problem of atherosclerosis were merely a matter of understanding the mechanism of cholesterol biogenesis, one would today indeed be entitled to optimism. Lately there has been considerable progress in the understanding of this biosynthetic process, and judging from the rate of advance, a knowledge of all the essential facts about cholesterol biogenesis now seems imminent. It will be useful to begin by pointing out the four major phases of cholesterol biogenesis which can be experimentally separated.

The first of these is the branching reaction which involves the coupling of three molecules of acetyl-CoA (acetyl coenzyme A) to β-hydroxy-β-methylglutaryl-CoA and its reduction to mevalonic acid (MVA). Over-all, these reactions involve the elimination of two molecules of water (or CoA) and the reduction of a carboxyl function. In the second stage, MVA molecules are polymerized to the hydrocarbon squalene, which is the immediate acyclic precursor of the steroids. This over-all transformation is also reductive and entails the uptake of two hydrogen atoms and the elimination of twelve molecules of water and of six molecules of carbon dioxide. Stages 1 and 2, which cover the reactions from acetic acid to squalene, are thus anaerobic in nature, and it has indeed been shown by Bucher (3) that in isolated liver, kept under strictly anaerobic conditions, the transformations of acetic acid stop at the squalene stage. All subsequent reactions are intimately associated with the oxidative metabolism of the tissues because the various oxygen atoms which enter in the course of cholesterol synthesis have their origin in molecular oxygen. This is true for the cyclization of squalene, which is oxidative (24) and for the oxidative removal of the three branched methyl groups of lanosterol (15). One may estimate that the biogenesis of cholesterol comprises altogether 20–30 separate enzymatic steps (2), only a few of which can now be studied at the enzymatic level. It is perhaps not surprising that the early steps which are concerned with transformations of soluble intermediates have yielded more readily to enzymatic analysis than the conversions beyond squalene.

The transformation of acetyl-CoA to acetoacetyl-CoA initiates the synthesis of both the sterols and of the higher fatty acids, but thereafter the two pathways diverge. Reduction to β-hydroxybutyric acid directs

the 4-carbon precursors towards the higher fatty acids, while the coupling with a third molecule of acetyl-CoA affords the branched-chain building stones for terpene and steroid biogenesis (18). This separation of pathways applies not only to the molecular changes but also to the intracellular distribution of the enzymes. Beta-hydroxybutyric acid formation takes place in the soluble part of the cytoplasm (11), whereas the condensation to hydroxymethylglutaryl-CoA is catalyzed by microsomal enzymes (18). Recently Rudney and his collaborators (7) have succeeded in closing the gap between hydroxymethylglutaryl-CoA and MVA by demonstrating the enzymatic reduction of the thioester portion of the dicarboxylic acid. The reductive steps of stage 1, which I mentioned earlier, thus refer to the conversion of a CoA ester first to the aldehyde stage and then to the stage of the primary alcohol.

Mevalonic acid, which was first isolated as the acetate-replacing growth factor for *Lactobacillus casei* (27), is now firmly established as an effective and presumably obligatory terpene and sterol precursor (22). Comparing the structure of mevalonic acid with the structural subunits of squalene, one is tempted to look upon mevalonic acid as an incipient isoprene. Elimination of two molecules of water and removal of the carboxyl group will afford the substituted butadiene without change in oxidation state, and this is, in fact, what appears to happen enzymatically. Working with soluble extracts of autolyzed yeast, we have shown a requirement for ATP (adenosinetriphosphate) in the early stages of the MVA-squalene conversion (1). Subsequently, Tchen (23) was able to demonstrate that ATP interacts with MVA to form a monophosphate ester which is relatively stable to acid and alkali, and therefore in all likelihood is the ester of a primary alcohol (MVA-5-phosphate). The further transformation of MVA-monophosphate, as determined either by the loss of the carboxyl group (C-1), or by squalene formation, requires another reaction with ATP, affording what appears to be a diphosphate of MVA (16). The two phosphorylation steps may be viewed as a means of facilitating the two dehydration steps by elimination of phosphate anion rather than of OH^-. Our indications are that the immediately ensuing step is the removal of the carboxyl group, possibly with the concurrent elimination of a phosphate residue. While we lack direct evidence as to the structure of additional intermediates between MVA and squalene, the outcome of various experiments with heavy hydrogen as a tracer has encouraged us to formulate the mechanism for squalene synthesis in considerable detail (17).

I have pointed to the fact that the terminal carbon atom of MVA (C-5) is reduced, and it seemed important to us to ascertain whether this state of reduction persists throughout the synthesis of the poly-

isoprenoid chain. By preparing 2-C^{14}-5-di-T-MVA, we were in a position to determine whether any hydrogen bound to C-5 is lost in the course of squalene formation. Had the hydroxymethyl group been oxidized either to aldehyde or to carboxylic acid, half or all of the carbon-bound tritium should have been lost. Analysis of the squalene derived from the doubly labeled MVA showed, however, that neither was the case (1), but that, in fact, all of the labeled hydrogen was retained. This result was unexpected because in all known carbon-carbon interactions at least one reactant carries a carbonyl function. In squalene synthesis, on the other hand, we seem to be dealing with the novel case of carbon-chain formation by condensation of two active methylene groups. Our results with doubly-labeled MVA and independent experiments with D_2O have led us to conclude that isoprene, presumably enzyme-bound, is formed by decarboxylation and phosphate elimination of MVA-diphosphate. According to our current views, three molecules of isoprene or isoprene-enzyme complex condense concertedly in a cation-initiated process to form a sesquiterpenoid intermediate (C-15). This can stabilize either by proton elimination to farnesene, by the uptake of OH^- to nerolidol or thirdly, by isomerization and OH^- uptake to farnesol. In this manner, the structures of the naturally occurring acyclic sesquiterpenes can be readily rationalized.

The hydrocarbon farnesene in turn provides an attractive structure for the reductive dimerization of two sesquiterpenoid units to squalene. This condensation also is formulated as a concerted process, a proton attacking one C-15 unit and a hydride ion the other (17). The mechanism which I have presented, while speculative, is the only one which accounts for two, in our opinion, significant results, one of which is the retention of hydrogen at C-5 of MVA and the other the limited uptake of deuterium (3–4 atoms) by squalene synthesized in a D_2O medium. We recognize the risk of attaching considerable weight to a few isotopic data, yet we feel that our scheme, even if proven wrong in detail, is correct in principle, and therefore useful as a working hypothesis. If squalene synthesis should occur largely by concerted mechanisms, many of the postulated intermediates are likely to elude isolation because of their transient nature. The same dilemma exists for the tetracyclization reaction by which squalene is convented to lanosterol. In their brilliant theoretical paper, Ruzicka and his collaborators (19) formulate the transformation of squalene to lanosterol as a "non-stop" reaction that is initiated by activated oxygen and leads to the steroid structure in a series of concerted electron displacements. Our studies on the enzymatic cyclization of squalene to lanosterol have given firm experimental support to these postulates, and we concur with the view that the tetracyclic

ring system is established without the stabilization of partially cyclized intermediates (25). The transformation of squalene to lanosterol entails a rearrangement of the carbon skeleton requiring an intramolecular shifting of either the methyl group at C-8 to the C-13 position or a shifting of two methyl groups to their adjacent position (from C-8 to C-14 and from C-14 to C-13). This important detail in the cyclization mechanism has now been settled in favor of the 1,2-methyl shift alternative (13), confirming the prediction by Eschenmoser *et al.* (6) that only a 1,2-methyl shift is consonant with a fully concerted cyclization mechanism.

Enzymatic studies on this interesting reaction, the system which we have referred to as the squalene-oxidocyclase system, have continued but have on the whole been disappointing. We appear to be facing here a problem familiar to students of steroid biogenesis and steroid transformations, namely, the fact that these enzymes are intimately associated with the microsomal particles. In our laboratory, efforts to solubilize either the cyclizing system or the enzymes concerned with the demethylation of lanosterol to cholesterol have so far remained without success. For the case of squalene cyclization by liver microsomes, we have established a requirement for molecular oxygen—this being the source of the 3-hydroxy group—for a soluble enzyme which has been fractionated to some extent and may be a triphosphopyridine nucleotide (TPNH) oxidase, and finally for a heat-stable, but so far elusive, cofactor (4). Here, as in other cases of enzymatic oxygenation, the mechanism of oxygen activation remains obscure. It is worth noting that in animal tissues the squalene molecule cyclizes exclusively to lanosterol, i.e., in an asymmetric manner. In plants, the same acyclic precursor undergoes cyclizations in much greater variety, viz., to pentacyclic triterpenes, symmetrical tetracyclic products, and to sterols as well.

Lanosterol is a short-lived intermediate and rapidly undergoes oxidative demethylation to cholestane derivatives, at least in liver. That the removal of the methyl groups at carbon atoms 4 and 14 is oxidative follows from the fact that these substituents are attached to quaternary carbon atoms. Therefore, structural considerations alone require an initial attack by oxygen. In line with this contention, we find that lanosterol is metabolically inert under anaerobic conditions and furthermore that there is stepwise methyl group oxidation by way of hydroxymethyl compounds, aldehydes, and carboxylic acid with eventual loss of CO_2 to the corresponding nor- compounds (15). Last year we isolated and described an intermediate in the lanosterol-cholesterol conversion to which the partial structure of a 14-norlanostadienol was assigned (8). By synthesizing the appropriate reference compounds, we have now been able to identify this C-29-sterol as $\Delta^{8,24}$-4,4-dimethyl-

cholestadienol (9). Thus, the double bonds remain in the 8-9 position during the first demethylation step. The elimination of the two methyl groups remaining at C-4 also involves successive oxidations, i.e., first an attack upon one methyl group, leading upon its removal to 4-monomethyl cholestane derivatives, observations in line with recent reports on the occurrence of 4α-methyl sterols in various natural sources (5, 14, 26). Studying the demethylation of lanosterol, we noted that some of the intermediates had the properties of ketones. We therefore prepared various sterols labeled with T (tritium) in the 3α position in order to localize the stage at which the 3-hydroxy group is oxidized. On enzymatic conversion of 3α-T-lanosterol or of 3α-T-14-norlanosterol to cholesterol the tritium was quantitatively lost. On the other hand, labeled hydrogen was fully retained during the conversion of 3α-T-zymosterol to cholesterol, and it therefore follows that the 3-hydroxy → 3-keto transformation takes place after the first of the three methyl groups has been removed from lanosterol. A confirmatory result is that 4,4-dimethyl-$\Delta^{8,24}$-cholestadiene-3-one is a precursor of cholesterol, while lanostadienone is not (12). If 4-carboxy sterols are intermediates, as we suspect, then the presence of a keto group at the 3 position, β to the carboxy group, can be looked upon as a means of facilitating decarboxylation. It can be argued, on the other hand, that the methyl group at C-14 is too distant from the 3-oxygen function, and hence that in this case the necessary activation is provided by the 8-9-double bond.

Zymosterol has the same relatively rare $\Delta^{8,24}$-double bond system as lanosterol, and it has therefore been logically regarded as the first fully demethylated cholesterol precursor. Zymosterol can be readily isolated from yeast and though the evidence that it is a normal constituent of animal tissues is not yet very strong, this sterol shows the metabolic activity expected from a normal cholesterol precursor (10, 20). The final structural alteration beyond zymosterol (assuming this to be an obligatory intermediate) cannot be formulated with any assurance except that the occurrence and metabolic activity of desmosterol, $\Delta^{5,24}$-cholestadienol (21), strongly point to the reduction of the side chain double bond as the last step in cholesterol biogenesis. It is worth noting that the over-all change from zymosterol to cholesterol, though it furnishes a more reduced product, nevertheless is dependent on molecular oxygen (10). As a mechanism for relocating the nuclear double bond from the 8-9 to the 5-6 position, a simple isomerization is therefore ruled out. The requirement for oxygen conceivably reflects the introduction of an additional hydroxy group in ring B, most likely in allylic

position, in which case the 5-6 double bond would be newly introduced and established by elimination of water.

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CHAPTER 2

Some Aspects of the Biosynthesis of Cholesterol from Mevalonic Acid

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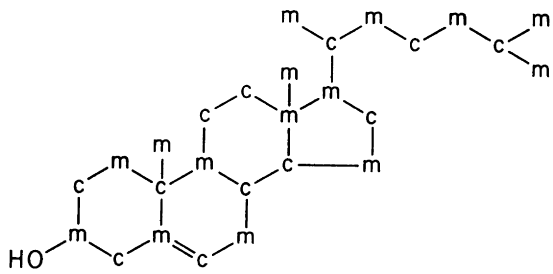
This report is the result of the joint effort of our team consisting of J. W. Cornforth, Rita H. Cornforth, Irene Youhotsky Gore, L. Gosselin, G. Popják, and A. de Waard. Detailed accounts of most of the experiments are contained in two papers to be published shortly in the *Biochemical Journal* (3, 9). Summaries of the results have already appeared (2, 6, 8).

When two years ago we completed our studies on the distribution of acetate carbons in the ring structure of cholesterol biosynthesized from C^{14}-acetate (4), we could assign every carbon atom in the sterol to either the methyl or the carboxyl carbon of acetate [formula (I)]. The pattern shown in formula (I) was in complete accord with the Woodward-Bloch hypothesis of cyclization of squalene to sterol.

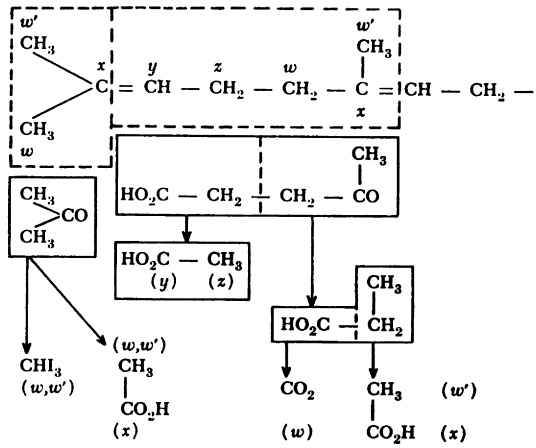


(I)

In spite of a great deal of experimental work trying to implicate branched chain C-6 and C-5 acids, such as 3-hydroxy-3-methylglutarate (HMG), 3-methylglutaconate, *iso*-valerate, 3-hydroxy*iso*-valerate, 3-methylcrotonate (dimethylacrylate), as the source of isoprenoid units used in the biosynthesis of squalene and sterol, no definite proof to this effect could be obtained. The situation was, however, changed very dramatically with the discovery of mevalonic acid (10) and identification of its structure as 3-hydroxy-3-methylpentano-5-lactone (13, 14). As you are all aware, the similarity of the structure of mevalonic acid (MVA) to that of HMG prompted Tavormina *et al.* (11) to test this

new substance as a precursor of cholesterol. Their discovery that DL-2-C^{14}-MVA was utilized for the biosynthesis of cholesterol in liver homogenates with an efficiency of about 40% made it very probable that at last the direct source of isoprenoid units had been found. We thought that a proof of this could be obtained in the surest way by ascertaining first whether squalene was also synthesized from MVA and if so, by determining the arrangement of the MVA-carbons in squalene.

The news of the discovery of Tavormina *et al.* (11) reached us in England early in November of 1956, but since the methods for the synthesis of MVA were not published at that time, we had to develop these for ourselves. Dr. and Mrs. Cornforth worked out the synthesis



SCHEME 1. Degradation of squalene into acetone and levulinic acid and into further fragments. Only the first two isoprenoid units from one-half of the squalene molecule are shown.

not only of MVA, but of all the anhydro compounds derivable from it; they were all labeled with C^{14} in position 2 and MVA in position 1 also. The anhydro compounds were made in order to test some of our ideas on the possible transformations of MVA during the biosynthetic reactions; I will discuss briefly the experiments with these substances at the end of my communication. We have been able to confirm the results of Tavormina and associates (11) without any difficulty and to show that liver homogenates under anaerobic conditions synthesized only squalene from 2-C^{14}-MVA. Moreover, the efficiency of squalene synthesis from MVA anaerobically was as great as the synthesis of cholesterol aerobically. This result was very satisfying because it supported fully the view that squalene was an intermediate in sterol bio-

synthesis, and it also enabled us to prepare a large batch of C^{14}-squalene for chemical degradation.

We degraded the sample of squalene biosynthesized from 2-C^{14}-MVA by ozonolysis as described previously (5). Acetone, levulinic acid, and succinic acid are the principal products of ozonolysis of squalene; of these only acetone and levulinic acid can be relied upon as arising from carbon atoms predicted by theory. The levulinic acid contains all five types of carbon atoms of the isoprenoid units of squalene, and consequently its carbon-by-carbon degradation gives an answer as to the arrangement of isotopic carbon in squalene (Scheme 1).

TABLE I

DISTRIBUTION OF C^{14} IN SQUALENE BIOSYNTHESIZED FROM 2-C^{14}-MEVALONIC ACID

Compounds and fragments analyzed	Specific activity of total carbon counts/min. at infinite thickness (A)	Molar specific activity[a] ($A \times n$)	
Squalene	788 ± 39	3940 × 6	
Acetone ($w + x + w'$)[b]			
Methyl carbons (w, w')	1967 ± 98	1967	4047
Acetic acid ($w + x$; $w' + x$)	1040 ± 52	2080	
Levulinic acid ($w' + x + w + z + y$):			
4-Aminopentanoic acid	777 ± 39	3890	
Acetic acid(1) ($z + y$)	0	0	
Propionic acid ($w' + x + w$)	1323 ± 66	3975	
COOH of propionic acid (w)	3880 ± 194	3880	
Acetic acid(2) ($w' + x$)	0	0	

[a] This was obtained by multiplying the values in Column A by the number (n) of carbon atoms contained in the compound analyzed. The molar specific activity of squalene is given as a multiple of 6 since squalene contains 6 isoprenoid units.

[b] The letters in parentheses indicate the carbon atoms of isoprenoid units (cf., Scheme 1).

The results of such degradation are shown in Table I. It is seen that the molar specific activity of the acetone was equal to one-sixth of the molar specific activity of the squalene. Since the specific activity of the acetic acid, obtained from the acetone after the iodoform reaction and which contained equally carbons ($w + x$) and ($w' + x$), was one-half of that of the methyl carbons of acetone (w, w'), carbon atom x contained evidently no C^{14}. The radioactivity of acetone is therefore either distributed between w and w' or is contained in only one of these carbons.

The levulinic acid, which was assayed both as the 2,4-dinitrophenyl-