EDGAR LEDERER

The Origin and Function of Some Methyl Groups in Branched-Chain Fatty acids, Plant Sterols and Quinones

By EDGAR LEDERER*

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Imagination is more important than knowledge Albert Einstein

The title of this lecture shows clearly the three topics treated: we shall first give a summary of the various ways invented by living cells for producing methyl-branched fatty acids, the most conspicuous being *C*-methylation with methionine and incorporation of propionic acid.

We shall then inquire about the origin of the 'extra methyl group' of the C_{28} sterols, e.g. ergosterol, of fungi, and of the 'extra ethyl group' of the C_{29} sterols, e.g. β -sitosterol, of higher plants. We shall learn that in the *C*-methylation leading to tuberculostearic acid and to ergosterol, contrary to expectation, only two of the three hydrogen atoms of the methyl group of methionine are transferred, and we shall also learn that both carbon atoms of the ethyl side chain of the C_{29} phytosterols come from methionine. We shall speculate about the mechanism of these *C*-methylations.

In the third part of this lecture, we shall inquire about the biological function of the methyl side chain of vitamin K and the ubiquinones, and we shall see that schemes can be devised that suggest an essential function of this methyl group in oxidative phosphorylation and perhaps in other biological reactions of these compounds.

Biosynthesis of methyl-branched fatty acids

Four different mechanisms that can lead to methyl-branched fatty acids are known: (1) *C*-methylation with the participation of methionine; (2) incorporation of propionic acid; (3) incorporation of the branched chains of leucine and iso-leucine, leading to the iso and anteiso acids respectively; (4) incorporation of mevalonic acid.

The first three mechanisms are well documented, principally in bacterial lipids, and the second and third also operate, to a limited extent, even in higher animals; the fourth has been observed in bacteria, higher plants and animals.

(1) *C*-Methylation with the participation of methio-

* Address: Institut de Chimie des Substances Naturelles, C.N.R.S., Gif-sur-Yvette, Seine-et-Oise, and Institut de Biochimie, Faculté des Sciences, Orsay, Seine-et-Oise, France.

nine. The *C*-methylation of numerous phenolic and terpenoid compounds by transmethylation from methionine was first discovered by Birch, English, Massy-Westropp, Slaytor & Smith (1958*a*) and Birch *et al.* (1958*b*) and proved in a brilliant series of investigations (for reviews see Birch, 1957, 1962).

In the field of methyl-branched fatty acids, *C*-methylation from methionine was first described by Lennarz, Scheuerbrandt & Bloch (1962), who showed that in *Mycobacterium phlei* [^{14}C]tuberculostearic acid (10-methylstearic acid) (I) is formed

from oleic acid in the presence of [$Me-^{14}C$]methionine. In this work, Lennarz *et al.* (1962) have proved the sequence:

Methionine is also the methyl donor for a methyl-branched acid isolated from the antibiotic variotin (Tanaka & Umezawa, 1962*a, b*). In our Laboratory, the origin of the *C*-methyl group of tuberculostearic acid from methionine has been confirmed with the human avirulent strain $H_{37}R_a$ of *M. tuberculosis* (M. Gastambide-Odier, unpublished work).

It is very remarkable that in lactobacilli, *Escherichia coli* and many other bacilli a closely related reaction leads from monounsaturated acids, such as vaccenic acid (*cis*-octadec-11-enoic acid), to cyclopropane acids, such as lactobacillic acid (*cis*-11,12-methyleneoctadecanoic acid) (II), or its

lower C_{17} homologue. Here, too, methionine is the C_1 donor (Hofmann & Liu, 1960; O'Leary, 1959; Chalk & Kodicek, 1961).

The question at once arises: can a 9,10-methylene acid (III) formed from oleic acid be a precursor of tuberculostearic acid (I) (see Scheme 1)? Some time ago, we tried to answer this question by incu-

bating a ^{14}C -labelled 9,10-methyleneoctadecanoic acid (dihydrosterculic acid) (III) with *M. phlei*. The fatty acids were then extracted and their methyl esters separated by gas-liquid chromatography. The tuberculostearate fraction was found to be devoid of radioactivity (R. E. Noble & D. Mercier, unpublished work). This negative result did not discourage us and we decided to approach the problem by another route. If a cyclopropane derivative is a precursor of tuberculostearic acid, then the methyl group of the latter should contain only two of the hydrogen atoms of the original methyl group of methionine. The formation of the cyclopropane fatty acids was studied in detail by Law, Zalkin & Kaneshiro (1963) and Pohl, Law & Ryhage (1963). The latter used $[\text{Me-D}_2]$ methionine and found that, as expected, two deuterium atoms are incorporated into a C_{17} cyclopropane acid. They proposed the mechanism shown in Scheme 2 by analogy with the reaction of a sulfoxonium methylene described by Corey & Chaykovsky (1962).

Dr Law, having come to Gif-sur-Yvette for a few months, and encouraged by results on ergo-

Scheme 1. Possible biosynthesis of tuberculostearic acid from dihydrosterculic acid.

sterol biosynthesis discussed below, grew *M. smegmatis* in the presence of $[\text{Me-D}_2]$ methionine and isolated labelled tuberculostearic acid.

Mass spectrometry showed unambiguously that only two deuterium atoms were incorporated (Jauréguiberry, Law, McCloskey & Lederer, 1964). (The small peak at m/e 315 is completely accounted for by naturally occurring ^{13}C in the molecular ion.) Further, the extensive work of Ryhage & Stenhagen (1960*a, b*) on mass spectrometry of fatty acids enabled us to locate the deuterium within the molecule. Cleavage of bonds on either side of the branched carbon atom gives rise to peaks (m/e 171 and 199) with a mass difference due only to C-10 and the attached methyl group. The increase of this difference by two mass units (m/e 171 and 201) in the spectrum of the deuterated compound thus shows that the two deuterium atoms are located in this group (Fig. 1). Since non-labelled tuberculostearate is also present, all peaks due to fragments containing the methyl group appear as doublets (e.g. m/e 199 and 201, and 269 and 271) when compared with the spectrum of the non-labelled ester.

This result prompted us to reinvestigate in more detail the possible role of dihydrosterculic acid (III) as a precursor of tuberculostearic acid. Other considerations pertinent to this problem are discussed below (see p. 456). For some time tuberculostearic acid was thought to be the only lipid of mycobacteria formed by C-methylation. More recently, evidence has been obtained in Gif-sur-Yvette that the methyl carbon atom of methionine can also be incorporated into mycolic acids ('high molecular β -hydroxy acids with a long side chain in α' ') (Asselineau & Lederer, 1960); the biosynthesis of these compounds by the condensation of several molecules of long-chain fatty acids has been reported (Gastambide-Odier & Lederer, 1960; Lederer, 1961*a, b*); from unpublished work by

Scheme 2. Mechanism for formation of cyclopropane acids proposed by Pohl *et al.* (1963).

M. Gastambide-Odier it now seems that one of the mycolic acids of the human avirulent strain H₃₇R_n of *M. tuberculosis* contains a cyclopropane group derived from methionine.

Moreover, studying the biosynthesis of the mycolic acids of *M. smegmatis*, Etémadi & Lederer (1964) observed the incorporation of ^{14}C from labelled methionine. Etémadi, Okuda & Lederer (1964) propose the structure (IV) for the α -smegmamycolic acids, where the carbon atom of the lateral methyl group comes from methionine (and the carboxyl-terminal 24 carbon atoms from tetracosanoic acid).

(2) *Incorporation of propionic acid.* After suggestions by Woodward (1956) and Gerzon *et al.* (1956) it was proved by Corcoran, Kaneda & Butte (1960) and Kaneda, Butte, Taubman & Corcoran (1962), as well as by Grisebach, Achenbach & Grisebach (1960) and Grisebach, Hofheinz &

Achenbach (1962), that the branched chain of erythronolide, the macrocyclic lactone moiety of the antibiotic erythromycin, is formed *in vivo* by the condensation of several molecules of propionic acid. The same pathway has been shown by Birch *et al.* (1960) for methymycin, by Grisebach & Achenbach (1962) and by Gilner & Srinivasan (1962) for magnamycin etc. Kaneda & Corcoran (1961), as well as Grisebach *et al.* (1962), have shown that, as expected, methylmalonic acid is the active form of propionic acid (for a review see also Grisebach, 1964).

We have found in Gif-sur-Yvette that the 'propionic acid mechanism' is also operative in the biosynthesis of several methyl-branched lipid compounds of mycobacteria. From experiments by Gastambide-Odier, Delaunéy & Lederer (1963*a*) it seems that phthiocerol (*Va*) is formed by the condensation of a C_{20} precursor with propionic

*C from C-3 of propionic acid; °C from methionine.

acid. An analogous structure is found in the 'phenol-glycol B' (Vb), the aglycone of mycoside B (Demarteau-Ginsburg & Lederer, 1963). Here, also, propionic acid serves as precursor for the branched end of the molecule (M. Gastambide-Odier & P. Sarda, unpublished work).

Mycocerosic acid (VI) is apparently produced by the condensation of a normal C₂₀ fatty acid with

four mol. of propionic acid (Gastambide-Odier, Delaumény & Lederer, 1963b). The phthienoic acids (VII), which are produced only by virulent strains, are most probably built up in a similar way.

Fatty acids derived from propionic acid seem not yet to have been found in higher plants. In the animal kingdom, however, representatives with one or several methyl branchings are known, which have been proved to be derived from propionic acid, namely α-methylbutyric acid and α-methylvaleric acid from the nematode *Ascaris lumbricoides*

(Saz & Weil, 1960, 1962), and the 2,4,6,8-tetramethyldecanoic acid (VIII) of the preen gland of

the goose, first isolated by Murray (1962). The structure (VIII) of this acid has been confirmed by Odham (1963); its biosynthesis from propionic acid was reported by Noble, Stjernholm, Mercier & Lederer (1963).

(3) *Biosynthesis of iso and anteiso acids.* Iso and anteiso acids with 15–17 carbon atoms are principally produced by bacteria (Akashi & Saito, 1960; Macfarlane, 1961; Thorne & Kodicek, 1962d).

Lennarz (1961) has studied in detail the role of isoleucine in the biosynthesis of 12-methyltetradecanoic acid by *Micrococcus lysodeikticus* and he suggested the reaction sequence shown in Scheme 3. The analogous formation of the isomeric 13-methyltetradecanoic acid from leucine was also proved.

Horning, Martin, Karmen & Vagelos (1961) have described a system synthesizing long-chain fatty acids from rat adipose tissue which catalyses the synthesis of iso and anteiso long-chain fatty acids from iso and anteiso short-chain fatty acyl-CoA derivatives respectively.

(4) *Incorporation of mevalonic acid.* Although mevalonic acid was discovered by Skeggs *et al.*

Scheme 3. Biosynthesis of an anteiso acid by *Micrococcus lysodeikticus* (Lennarz, 1961).

(1956) as 'acetate-replacing factor' for lactobacilli, and despite its most important role in the biosynthesis of terpenoids and steroids in plants and animals, its function in bacteria is still obscure. Bacteria seem to be incapable of synthesizing sterols (for references see Asselineau & Lederer, 1960), and no authentic sterol has ever been isolated from bacteria grown on a synthetic medium. The traces of 'steroidal fractions' isolated from such bacterial cultures represent less than 0.001 % of their dry weight, whereas other living organisms contain 1.4–7 % of sterols (see Asselineau, 1962). Thorne & Kodicek (1962*a-c*) studied in detail the metabolism of mevalonate in lactobacilli and isolated a series of unsaponifiable unsaturated compounds. Mevalonate thus contributes very little to the bulk of the branched-chain fatty acids found in Nature, except for the apparently rare existence of 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid) derived from phytol (Klenk & Kahlke, 1963; Lough, 1963) and the prenoic acids studied by Christophe & Popják (1961).

Function of branched-chain fatty acids. In discussing the function of the branched-chain acids we realize that we are leaving the firm ground of experimental evidence. The most obvious difference between a straight-chain acid and a branched-chain acid is in the respective melting points. Except for the iso acids, which have practically the same melting point as the corresponding straight-chain acids, all branched-chain acids have lower melting points. If one assumes that a low melting point is a desirable feature for the metabolic functions of fatty acids, the branched-chain acids are certainly

more useful than the saturated ones; but now that we know that some of them at least are synthesized from unsaturated acids, which melt still lower, one can only wonder about the 'sense' of spending the precious methyl groups of methionine in that way.

The use of propionic acid for chain-lengthening and chain-branching is of obvious value in producing saturated low-melting fatty acids; and we may well express our admiration for the goose, which has 'chosen' such an elegant solution to the problem of producing a stable, low-melting lipid necessary for impregnation of its feathers, making them hydrophobic.

Biosynthesis of the C-24 side chain of phytosterols

The brilliant investigations of Bloch, Cornforth, Lynen, Popják and others (for reviews see Bloch, 1957; Lynen, Eggerer, Henning & Kessel, 1958; Cornforth, 1959, 1961; Arigoni, 1960; Popják & Cornforth, 1960) have elucidated the pathway of biosynthesis of sterols in yeast and higher animals. They have shown that the major steps lead from acetate via mevalonate to farnesol and then to squalene, which is cyclized to the C₃₀ precursor lanosterol. This then loses three methyl groups and undergoes isomerization to desmosterol (IX) (Stokes, Hickey & Fish, 1958), the immediate precursor of cholesterol (X) in the animal organism.

For some time it has been tacitly assumed that in higher plants the biosynthesis of sterols follows the same pathway (for an excellent review on plant steroids see Heftmann, 1963). In recent years several groups of workers have confirmed this; they have demonstrated the incorporation of radio-

activity from labelled acetate and mevalonate into plant triterpenes and phytosterols (Bennett, Heftmann, Purcell & Bonner, 1961; Nicholas, 1962; Baisted, Capstack & Nes, 1962; Gros & Leete, 1963; Johnson, Heftmann & Houghland, 1964). It thus seems fairly certain that the 27 carbon atoms of the skeleton of the phytosterols come from mevalonate, via lanosterol and (perhaps) desmosterol (IX). But what is the origin of the C-28 'extra methyl group' in ergosterol (XI), brassicasterol and their derivatives, and of the C-28 and C-29 'extra ethyl group' in β -sitosterol (XII), stigmasterol etc.?

'*Extra methyl group*' of the C_{28} sterols. Let us first study the origin of C-28 in ergosterol (XI), as the typical representative of the C_{28} plant sterols. In 1957 it was reported that formate can serve as a source of C-28 in the biosynthesis of ergosterol (Danielsson & Bloch, 1957) or of the C_{31} triterpene eburicoic acid (Dauben, Fonken & Boswell, 1957). In the same year Alexander, Gold & Schwenk (1957) showed that methionine was the direct donor of the C-28 methyl group of ergosterol, and Parks (1958), using a cell-free ergosterol-synthesizing system, found that *S*-adenosylmethionine was more efficient than methionine as a methyl donor.

Alexander & Schwenk (1957) concluded from experiments with ^{14}C - and tritium-labelled methionine that all three hydrogen atoms of the methyl group were transferred to ergosterol. This seemed in agreement with previous work of du Vigneaud, Rachele & White (1956), who, using intramolecularly labelled [$\text{Me-}^{14}\text{CD}_3$]methionine, had shown conclusively that the whole methyl group is transferred to the acceptor molecule in *N*-methylation leading to the biosynthesis of choline and creatine. In reality, the findings of Alexander & Schwenk (1957) are not conclusive. If one disregards the isotope effect of tritium and if only two hydrogen atoms were transferred, the T: ^{14}C ratio in ergosterol would be 66.6% [the T: ^{14}C ratio of the starting material (methionine) being taken as 100%]. Alexander & Schwenk (1957) found the ratio to be 86–91%. However, if the isotope effect of tritium is accounted for, these values can be considered to be in agreement with the transfer of only two hydrogen atoms. [The maximum isotope effect of T at 25° is approx. 55 (cf. Melander, 1960), i.e. the loss of the T atom can be 55 times slower than that of H. If one assumes only a very small isotope effect, e.g. 6, the theoretical value for the transfer of CH_3 , in the experiments of Alexander & Schwenk (1957), would be 92%.] In view of this uncertainty we decided to use [Me-D_3]methionine. A methionine-less strain of *Neurospora crassa* was grown for 4 days in the presence of [Me-D_3]methionine. The cells were harvested and saponified, and ergosterol was isolated from the unsaponifiable residue by gas-liquid chromatography. The

eluate from the column was submitted to mass spectrometry. The mass spectrum of 'normal' ergosterol (XI) shows the molecular peak at 396, with a strong peak at m/e 253 representing the tetracyclic ring system (which has lost H_2O and the side chain). The mass spectrum of ergosterol produced by *N. crassa* in the presence of [Me-D_3]methionine shows a molecular peak at 398 (no trace of a peak at 399 other than the ^{13}C isotope peak of m/e 398) and the peak at m/e 253 unchanged (Fig. 2). This proves that the extra 2 mass units are in the side chain, as expected (Jauréguiberry *et al.* 1964).

In these experiments it was thus found that only two hydrogen atoms of the methyl group of methionine are transferred with C-28 of ergosterol, in agreement with the similar result for the methyl group of tuberculostearic acid (Fig. 1). How can we explain these results? Several possibilities must be considered: (1) The CD_3 group of methionine in its activated form as *S*-adenosylmethionine is not stable and partly exchanges its deuterium with the water of the medium. This can be ruled out, as the above-mentioned experiments of du Vigneaud *et al.* (1956) showed the stability of the CD_3 group under conditions of *N*-methylation. Further, it would indeed be fortuitous if exactly one atom of deuterium were lost in this manner. (2) *S*-Adenosylmethionine is not the immediate precursor, but methylenetetrahydrofolate. This seems most improbable, as Kisliuk (1963) has shown that the transfer of a C_1 unit from methylenetetrahydrofolate to homocysteine to form methionine is irreversible. Moreover, the experiments of Alexander & Schwenk (1957) and of Parks (1958) showed that methionine is the most efficient donor for the biosynthesis of ergosterol. (3) A cyclopropane derivative is an intermediate. This is an attractive possibility, all the more so in view of the situation (discussed above) with lactobacillic acid (II) and tuberculostearic acid (I). *S*-Adenosylmethionine could thus lose one hydrogen atom to give an 'ylide' as source of a carbene, which would add to the double bond of the acceptor molecule (oleic acid or desmosterol) to give a cyclopropane derivative (a carbene could add to an unpolarized double bond; an ylide would add only to a polarized double bond); this would then be hydrogenated to the *C*-methyl compound, namely tuberculostearic acid (I) or ergosterol (XI). An isomerization of the cyclopropane derivative 24,25-methylenedesmosterol could lead (Scheme 4) to 24-methylencholesterol (XIII) (see below). We have recently speculated about different other possible mechanisms of formation of 24-methylencholesterol (XIII) (Hügel, Vetter, Audier, Barbier & Lederer, 1964).

Other hypotheses were suggested by Dr Bianca Tchoubar, during a discussion of our results:

(a) It is known that hydroxymethylation of $C=C$ double bonds occurs easily (Prins reaction: see Arundale & Mikeska, 1952; Yang, Yang & Ross, 1959). The formation of a hydroxymethyl derivative from a methylated sulfoxide or *N*-oxide has been described by Pummerer (1910) and Polonovski & Polonovski (1927). One could thus consider the possibility of the oxidation of the $R \cdot S^+(CH_3) \cdot R'$

group of adenosylmethionine via the sulfoxide $R \cdot S^+(CH_3) \cdot R'$ to the hydroxymethyl derivative $R \cdot S^+(CH_2 \cdot OH) \cdot R'$, which would be the active hydroxymethylating agent. After dehydration and reduction the newly formed methyl group would contain only two of the original hydrogen atoms of

Fig. 2. Mass spectra of (a) ergosterol and (b) $[D_2]$ ergosterol.

Scheme 4. Possible ways of formation of 24-methylenecholesterol. The three compounds in square brackets do not seem to have been detected yet.

methionine. (b) A more attractive possibility (Scheme 5) involves the existence of an ylide (B), leading to a precursor (D) common to the formation of both cyclopropane and *C*-methyl groups. In recent papers on 'model pathways for enzymatic demethylation', Oae, Kitao, Kawamura & Kitaoka (1963*a, b*) have postulated that dimethyl sulphoxide reacts with acetic anhydride to give the ylide intermediate (A). We might thus suggest the formation of an analogous ylide (B) by oxidation of *S*-adenosylmethionine. This would then react with a double bond (C), previously polarized by linkage with an enzyme, to give the intermediate (D). Reduction of (D) would give the *C*-methyl compound (E), whereas a cyclopropane derivative (F) could be formed by cyclization accompanied by liberation of the enzyme.

C₂₉ sterols. After some initial speculations (Robinson, 1955; Arigoni, 1959), the origin of the 'extra two carbon atoms' of the C₂₉ sterols of higher plants has been studied recently by several groups of authors. By analogy with the role of methionine as methyl donor in ergosterol biosynthesis, ethionine could have been assumed to be an ethyl donor for the biosynthesis of the C₂₉ sterols.

Scheme 5. Postulated (Oae *et al.* 1963*a, b*) ylide intermediate (A) in the reaction of dimethyl sulphoxide and acetic anhydride, and the reaction of a postulated analogous ylide (B) (formed by oxidation of *S*-adenosylmethionine) with a double bond (C) (polarized by linkage with an enzyme) to give an intermediate (D) that would give a *C*-methyl compound (E) on reduction or a cyclopropane derivative (F) on cyclization and liberation of the enzyme.

This rather improbable pathway has been ruled out by Castle, Blondin & Nes (1963), as well as by Nicholas & Moriarty (1963), who have made parallel incorporation experiments with ethyl-labelled L-ethionine and found much less incorporation than with methyl-labelled methionine.

Castle *et al.* (1963) seem to be the first to have published a detailed scheme showing the formation of the ethyl side chain of sitosterol (XII) from two C₁ units (Scheme 6). They grew pea seedlings (*Pisum sativum*) in the presence of L-[Me-¹⁴C]-methionine and isolated radioactive sitosterol. They had previously shown that in *Pisum sativum* radioactive mevalonate is predominantly incorporated into β-amyrin. Since there was no incorporation of labelled methionine into β-amyrin, they conclude that 'the radioactivity incorporated into the steroid cannot reasonably have arisen via the mevalonate route; consequently, carbon atoms numbered 1 through 27 inclusive should not be labelled. This leaves only C-28 and C-29 as reasonable positions for the radioactivity in agreement with the mechanism shown in the formulas'. Nicholas & Moriarty (1963) have reported similar experiments for β-sitosterol (XII) produced by *Salvia officinalis*.

Bader, Guglielmetti & Arigoni (1964) fed DL-[Me-¹⁴C]methionine to rhizomes of *Menyanthes trifoliata* and isolated radioactive spinasterol (XIV). Degradation showed that all the isotope was in the ethyl side chain and that both carbon atoms were equally labelled, thus proving conclusively that C-28 and C-29 come from methionine. These authors also consider a 24-methylene derivative as a possible precursor for the second methylation step (Scheme 7).

The interest of our own Laboratory in the origin of the side chain of phytosterols stems from work on 'queen substance', the pheromone of the queen bee (Barbier & Lederer, 1960; Pain, Barbier, Bogdanovsky & Lederer, 1962). During this study, the sterol of queen bees and worker bees was shown to be principally 24-methylenecholesterol (XIII) (Barbier & Schindler, 1959). As most insects are incapable of synthesizing sterols (Clark & Bloch, 1959*a, b*; see also review by Clayton, 1964), this bee sterol, which had previously been found only in some marine invertebrates (Idler & Fagerlund, 1955), could have been a transformation product of some plant sterol. We then showed that 24-methylenecholesterol (XIII) is rather easily isolated also from the pollen of various plants (Barbier, Hügel & Lederer, 1960). As bees feed on pollen, their 24-methylenecholesterol (XIII) is most probably directly of dietary origin.

More recently, we have analysed pollen sterols by mass spectrometry and have found (Table 1) that they are a mixture of C₂₇, C₂₈ and C₂₉ sterols with

one and two double bonds (Hügel *et al.* 1964). A similar series of sterols has also been found in the spores of the fern *Polystichum filix mas* (Dupéron, Vetter & Barbier, 1964) and in the cotyledons of *Phaseolus vulgaris* (Dupéron, 1964).

The C_{27} sterols are most probably desmosterol (IX) and cholesterol (X); the latter had already been found by Tsuda, Agaki, Kishida, Hayatsu & Sakai (1958) in red algae and by Johnson, Bennett & Heftmann (1963) in higher plants (*Solanum*

Scheme 6. Biosynthesis of plant steroids (Castle *et al.* 1963).

Scheme 7. Postulated biosynthesis of spinasterol (Bader *et al.* 1964).

Table 1. *Analysis of pollen sterols*The results are those of H $\ddot{u}$ gel *et al.* (1964).

	Sterol composition (% of total)		
	C ₂₇ sterols	C ₂₈ sterols	C ₂₉ sterols
Mixed pollen	15	50	35
Chestnut (<i>Castanea vulgaris</i>) pollen	3	23	74
Hazel (<i>Corylus avellana</i>) pollen	0	25	75

Scheme 8. Postulated biosynthesis of pollen sterols (H $\ddot{u}$ gel *et al.* 1964).

tuberosum and *Dioscorea spiculiflora*). The C₂₈ sterols are 24-methylenecholesterol (XIII) and probably campesterol (XV), or a stereoisomer (an ergost-5-en-3-ol). The C₂₉ sterols contain components with an ethylidene side chain, e.g. fucosterol (XVI), and with an ethyl side chain, e.g. β -sitosterol (XII). From this study, in which the simultaneous presence of all the members of a possible biosynthetic series had been demonstrated, the sequence shown in Scheme 8 was proposed.

For an experimental study of this scheme, we wished to use a fucosterol-producing algal strain. Contrary to our expectation, several strains of *Nitella* and *Nitzschia* grown on a synthetic medium

produced no fucosterol, but only C₂₈ and C₂₉ sterols with saturated side chains at C-24. Finally, the marine brown alga *Laminaria saccharina* was incubated in sea water at 15° for 1 week in the presence of [Me-¹⁴C]methionine; this gave a good yield of labelled fucosterol (XVI), which was then degraded. Ozonization gave acetaldehyde (Scheme 9), which was isolated as its 2,4-dinitrophenylhydrazone. This was oxidized to acetic acid, which was then split into its two C₁ fragments by the Schmidt reaction. The carboxyl carbon (C-28) gave 5100 counts/min. and the methyl carbon (C-29) gave 5700 counts/min., showing that both carbon atoms of the ethylidene group of fucosterol were equally labelled and come from methionines (Villanueva, Barbier & Lederer, 1964). This finding thus confirms and extends Arigoni's results (cf. Bader *et al.* 1964).

We have thus learnt new facts about the origin of the methyl group of the C₂₈ steroids and of the ethyl group of the C₂₉ steroids. But what is their function? Here the field is still open for the wildest speculations. There seem to be no investigations explaining the necessity or the use of these side chains for plant organisms. It is well known, however, that the animal organism is capable of distinguishing phytosterols from cholesterol. Let us mention only three aspects of this problem: (1) Phytophagous insects are very often obliged to transform plant sterols into cholesterol by eliminating the one or two extra carbon atoms, which they seem to dislike [see review by Clayton (1964); for a discussion of steric factors in relation to sterol side chains and the fitting of sterol molecules into a 'functional space' in insects, see Clayton & Bloch (1963)]. (2) It is known since the early work of Sch $\ddot{o}$ nheimer (1931) that plant sterols are only poorly absorbed by the intestine in comparison with cholesterol. Glover & Morton (1958) have reported that very little transfer of sitosterol from the lumen to the mucosa occurs and that it is the specificity of this transfer mechanism for sterols that is responsible for the non-absorbability of phytosterols. Swell, Trout, Field & Treadwell (1959) consider that the principal block in phytosterol absorption occurs within the mucosa, in the transfer from the mucosa to the lymph; this may be esterification by cholesterol esterase or chylo-

Scheme 9. Ozonization of fucosterol (Villanueva *et al.* 1964).

micron formation. For a discussion of the important problem of the possible hypocholesteraeamic activity of phytosterols, see Kritchevsky (1958). (3) Ergocalciferol (vitamin D₂ derived from ergosterol) is much less active in chicks than is cholecalciferol, the corresponding vitamin D₃ with the cholesterol side chain (Massengale & Nussmeier, 1930; see also Harris, 1954). A more detailed investigation of the numerous aspects of animal metabolism of phytosterols might well be rewarding.

Possible function of the 2-methyl group in vitamin K and the ubiquinones

In the first two parts of this lecture, we were more concerned with the origin of methyl groups than with their function. In this third part, we shall, on the contrary, be principally interested in the function of a particular methyl group in several natural quinones having important biological activities. Three principal groups of natural

2-methyl-3-oligoisoprenyl quinones are known: the vitamin K compounds (XVII); the ubiquinones (coenzyme Q) (XVIII); the tocopherol quinones (XIX) produced by oxidation of the tocopherols (vitamin E). [The 2-methyl group of the ubiquinones comes from methionine (Rudney & Parson, 1963; Olson *et al.* 1963). Nothing seems to be known about the origin of this group in the vitamin E and K series. In the plastoquinones (Kofler *et al.* 1959; Crane, 1959 *a, b*), which are involved in electron transport in the chloroplast, the 2-methyl group is missing. These compounds do not seem to be implicated in oxidative phosphorylation.] The 2-methyl group seems essential for the biological activity of all these compounds; in the first two groups, the double bond in the isoprene residue nearest to the quinone ring is also essential (Brodie, 1961).

Let us first consider the situation with vitamin K. The simplest compound having vitamin K

activity is menadione (2-methyl-1,4-naphthoquinone). All other vitamin K-active compounds possess this 2-methyl group (Fieser, Tishler & Sampson, 1941). From the work of Martius & Esser (1958) and Billeter & Martius (1960) it seems that menadione is only a provitamin K and that the animal organism 'hooks on' an unsaturated isoprenoid side chain, thus producing vitamin K₂-20 for instance.

We try to show below that both essential features, the 2-methyl group and the $\beta\gamma$ -double bond in the side chain, co-operate in the formation of the methylenequinone chromane (XX), which seems to be a key substance for the biological activity of these compounds. The discovery of the formation of this category of compounds stems from attempts to explain the role of quinol phosphates in oxidative phosphorylation. Clark, Hutchinson, Kirby & Todd (1961) were the first to

show that the oxidation of quinol phosphates (XXI) generates 'active metaphosphate', which reacts with AMP to form ADP, or with inorganic phosphate to give pyrophosphate (Scheme 10). Since quinones of the vitamin K group and the ubiquinones have been suggested as possible intermediates in oxidative phosphorylation (Brodie & Ballantine, 1960*a, b*; Brodie, 1961; Asano, Brodie, Wagner, Wittreich & Folgers, 1962*a, b*; Gruber, Höhl & Wieland, 1963), the question must be answered how, *in vivo*, a quinol phosphate might be formed from a quinone, without participation of an energy-rich compound.

A simple mechanism (Scheme 11), involving a 1,2-addition of phosphate to one of the carbonyl groups of the quinone, has been proposed by Wieland & Pattermann (1959). Clark & Todd (1961) have suggested a participation of the $\beta\gamma$ -double bond of the side chain, placing the phos-

Scheme 10. Oxidation of quinol phosphates to generate 'active metaphosphate', which subsequently reacts with AMP to form ADP or with inorganic phosphate to form pyrophosphate (Clark *et al.* 1961).

Scheme 11. Mechanism for the formation of quinol phosphates proposed by Wieland & Pattermann (1959).

phate on C-4 instead of C-1. Chmielewska (1960) has proposed another mechanism (Scheme 12), involving the double bond of the side chain and 1,2-addition of phosphate to a methylenequinone.

Brodie (1961), Russel & Brodie (1961) and Asano *et al.* (1962*a, b*) have obtained evidence for the enzymic formation of a 6-chromanylphosphate (XXII) from vitamin K as an intermediate in oxidative phosphorylation.

(XXII)

More recently, Vilkas & Lederer (1962) have modified the scheme of Chmielewska (1960) by suggesting a 1,4-addition instead of a 1,2-addition of phosphate to a methylenequinone chromane (Scheme 13).

Calculations of differences of bond energies show that the isomerization of a quinone to a methylenequinone is considerably favoured by the simultaneous cyclization of the unsaturated side chain to a chromane. Thus, in the reactions shown in

Scheme 14, one must supply 20.3 kcal./mole to (XXVI) to isomerize it to the methylenequinone (XXVI*a*), whereas (XXVII) and (XXVII*a*) have nearly equal stabilities (Vilkas & Lederer, 1962).

The mechanism shown in Scheme 13 was first presented at the IUPAC meeting in Brussels, June 1962, and published in December 1962 in *Experientia*. Unlike several other speculations on oxidative phosphorylation, it has rapidly received experimental proof, at least in part.

In May 1963, Folkers and his group published two preliminary communications (Wagner *et al.* 1963*a*; Erickson, Wagner & Folkers, 1963) suggesting the formation of a methylenequinone chromane (XX) by the following reactions: (1) Vitamin K₁-20 (XVII) was treated with sulphuric acid to give the γ -hydroxyquinone (XXVIII); this then reacted with acetyl chloride giving the 5-chloromethyl-6-chromanyl acetate (XXIX) (Scheme 15); similarly, the γ -hydroxyquinone derived from ubiquinone-20 gave with acetyl chloride the corresponding 5-chloromethyl-6-chromanyl acetate. (2) During this acid treatment, vitamin K₁-20 also gave an oily compound to which Wagner *et al.* (1963*a*) ascribed the structure of the dimer (XXX) formed from two molecules of the methylenequinone (XX). In the meanwhile, in our Laboratory, Mamont, Azerad, Cohen, Vilkas & Lederer (1963) had also investigated the dimerization reaction and described the conversion of vitamin K₁-20 in perchloric acid-acetic acid into a dimer obtained as yellow crystals, m.p. 77–79°. The infrared-absorp-

Scheme 12. Mechanism for the formation of quinol phosphates proposed by Chmielewska (1960).

Scheme 13. Mechanism for the formation of quinol phosphates proposed by Vilkas & Lederer (1962). Reaction (1): vitamin K (XVII) or a ubiquinone (XVIII) is isomerized to a methylenequinone chromane (XX). Reaction (2): the methylenequinone adds phosphate at the 1,4-positions to give the benzyl phosphate (XXIII) (such 1,4-additions to *o*- or *p*-methylenequinones are well known; Hultzs, 1948; Martin, 1956). Reaction (3): an intramolecular migration places the phosphate on the phenolic hydroxyl group. Reaction (4): reduction of the benzyl alcohol (XXIV) thus produced gives the chromanyl phosphate (XXII) described by Asano *et al.* (1962*a, b*) and considered to be an intermediate in oxidative phosphorylation. Reaction (5): the chromanyl phosphate isomerizes to the quinol phosphate (XXV). Reaction (6): the quinol phosphate is oxidized to give 'active metaphosphate' and the quinone, as proposed by Clark *et al.* (1961). For more details and for alternative possibilities, see Vilkas & Lederer (1962) and Clark (1963).

Scheme 14. Isomerization of quinones to methylenequinones. For reaction (1), $\Delta H_{(XXVI)-(XXVIa)}$ is 20.3 kcal./mole; for reaction (2), $\Delta H_{(XXVII)-(XXVIIa)}$ is 0.3 kcal./mole (Vilkas & Lederer, 1962).

Scheme 15. Formation of 5-chloromethyl-6-chromanyl acetate (XXIX) from vitamin K₁-20 (a) by treatment with sulphuric acid to give the γ -hydroxyquinone (XXVIII), which then reacted with acetyl chloride (Wagner *et al.* 1963a; Erickson *et al.* 1963), or (b) directly by treatment with acetyl chloride in perchloric acid (P. Mamont, R. Azerad, P. Cohen, E. Vilkas & E. Lederer, unpublished work).

naphthachromanol (XXXI) prepared by reduction and cyclization of vitamin K₁-20.

A further confirmation of the existence of the methylenequinone was obtained by treating vitamin K₁-20 with acid in the presence of an excess of styrene (Scheme 17). After careful chromatography of the reaction mixture, a yellow oil was obtained: the elementary analysis and the nuclear-magnetic-resonance and mass spectra of this compound were in agreement with the structure of the condensation product (XXXII) (Mamont *et al.* 1963). There seems to be no doubt that these reactions, reported independently from two Laboratories, prove a ready isomerization of 2-methyl-3-oligoisoprenylquinones to 2-methylenequinone chromanes. Cameron, Scott & Todd (1964) have shown that such isomerizations can also occur easily with simpler methyl-substituted quinones.

Dimerization of α -tocopherol and its derivatives has been described by Schudel, Mayer, Metzger, Rüegg & Isler (1963). Here, too, the intermediate formation of a methylenequinone is considered to occur.

The preparation of a 5-chloromethyl-6-chromanyl acetate (XXIX) described by Wagner *et al.* (1963a, b) also provides support for reaction (2) in Scheme 13, the 1,4-addition to the methylenequinone to give a benzyl alcohol derivative.

P. Mamont, R. Azerad, P. Cohen, E. Vilkas & E. Lederer (unpublished work) have now obtained the 5-chloromethyl-6-chromanyl acetate (XXIX) directly from vitamin K₁-20 by treatment with acetyl chloride in perchloric acid, in a yield of 70 % (Scheme 15). By treatment of the 5-chloromethyl-6-chromanyl acetate (XXIX) with silver dibenzyl

tion and nuclear-magnetic-resonance spectra of this pure compound are in agreement with formula (XXX) (Scheme 16). Moreover, the same dimer is obtained by oxidation by ferricyanide of the

Scheme 16. Dimerization of vitamin K₁-20 (a) directly in perchloric acid-acetic acid or (b) by oxidation of the naphthochromanol obtained by reduction and cyclization of the vitamin (Mamont *et al.* 1963).

Scheme 17. Reaction of vitamin K₁-20 with styrene (Mamont *et al.* 1963).

phosphate, Wagner *et al.* (1963*b*) obtained, after debenzoylation, the 5-phosphomethyl-6-chromanyl acetate (XXXIII) (Scheme 18), which they consider to be 'more productive than previously reported hydroquinone [quinol] monophosphates and 6-chromanyl phosphates for studies of biological oxidative phosphorylation'. These authors consider the possibility that the oxidation of the benzyl phosphate might give 'active phosphate', eliminating the necessity of the migration of the phosphate group (reaction 3 in Scheme 13) (Erickson *et al.* 1963). This point awaits further experimental study, all the more so since the existence of other phosphorylated energy-rich intermediates, e.g. phosphohistidine (Boyer *et al.* 1963) or a phosphorylated NAD derivative (Griffiths, 1963) has been demonstrated in recent work on oxidative phosphorylation.

Let us finally examine another aspect of the methylenequinone problem.

Scheme 18. Synthesis of 5-phosphomethyl-6-chromanyl acetate (Wagner *et al.* 1963*b*).

If such compounds really operate in oxidative phosphorylation, then structurally similar compounds should act as inhibitors.

Howland (1963) studied the uncoupling and inhibiting properties of 3-alkyl-2-hydroxy-1,4-naphthaquinones, and found that those that possess a 2',3'-unsaturated side chain at the 3-position (e.g. lapachol and lomatiol) appear to uncouple oxidative phosphorylation, whereas those that are saturated at this bond (e.g. hydrolapachol) act as phosphorylation inhibitors, in much the same manner as oligomycin. Howland (1963) concludes that his results are also in favour of the involvement of chromanes in oxidative phosphorylation.

One of the most potent inhibitors of oxidative phosphorylation in whole mitochondrial and in submitochondrial particles is atractyloside (Bruni, Contessa & Luciani, 1962; Vignais, Vignais & Stanislas, 1962; Contessa & Fassina, 1963; Vignais & Vignais, 1964), a hypoglycaemic drug, the structure of which (XXXIV) has been established by Ajello, Piozzi, Quilico & Sprio (1963). [This hypoglycaemic action could be explained by its activity as antagonist of oxidative phosphorylation (inhibition of mobilization of hepatic glycogen).]

Hydrogenation of the methylene group abolishes this activity (A. Quilico, personal communication), thus showing that at least one of the 'active sites' of atractyloside is the methylene group.

During a recent visit to Gif-sur-Yvette, Dr V. M. Clark suggested to us that atractyloside, the aglycone of which is a homoallylic alcohol, could easily form the corresponding cation by elimination of the hydroxyl group and would thus become capable of adding phosphate. Vignais & Vignais (1964) have come to the conclusion that atractyloside acts by combining reversibly with a phosphorylated energy-rich intermediate.

Another hypoglycaemic compound, hypoglycine (XXXV), also has a methylene group (activated by the adjacent cyclopropane ring). De Ropp *et al.* (1958) state that reduced hypoglycine is inactive and that oxidative phosphorylation is inhibited in liver mitochondria from hypoglycine-treated rats.

A great many antibiotics and toxic compounds contain actual or potential methylenequinone or methyleneketone structures: Dr P. Vignais and Dr P. Vignais-Daumas (Grenoble) have kindly agreed to test several of these and have found that episclerotiorin (XXXVI) inhibits oxidative phosphorylation at a concentration of 50 $\mu\text{g./ml.}$ (i.e. 0.1 mM); citrinin (XXXVII) at the same concentration is inactive, whereas sarcomycin (XXXVIII) was inhibitory at a concentration of 1 mM. This study will be extended and should provide some useful information about the mechanism of action of several of these compounds.

Let us now leave the particular problem of oxidative phosphorylation and reconsider vitamin K, which acts in higher animals as a specific catalyst for prothrombin synthesis. Here, again, two principal structural features are necessary: the 2-methyl group and the β -double bond in the 3-isoprenoid side chain (Fieser *et al.* 1941). We thus expect that, even if prothrombin synthesis is not directly linked to oxidative phosphorylation, a methylenequinone chromane will be shown to be the active form. This active form could add to thiol groups of enzymes (for addition of thiols to methylene compounds, see review by Larsson, 1962) or

Episclerotiorin
(XXXVI)

Citrinin
(XXXVII)

Sarcomycin
(XXXVIII)

other active groups or compounds, other than phosphates.

We expect that the near future will bring most interesting developments in this field. To quote Louis Pasteur: 'It is characteristic of Science and Progress that they continually open new fields to our vision'.

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