Some Problems Concerning Biological *C*-Alkylation Reactions and Phytosterol Biosynthesis

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I: Some General Problems Concerning *C*-Alkylation *in vivo*

C-Methylation plays a great rôle in the biogenesis of biologically important quinones, such as the ubiquinones, the tocopherols, the plastoquinones, phylloquinone, the menaquinones, which have been one of the major and most successful research interests of Professor R. A. Morton;[1] this topic thus seems particularly appropriate for the present lecture.*

The transfer of the methyl group of methionine to an unsaturated carbon atom resulting in a methyl side chain was first postulated by Birch *et al.*[2] in 1954, and was then demonstrated experimentally in the case of mycophenolic acid (1)† (Birch *et al.*[3]).

At about the same time Alexander *et al.*[4] showed that the 'extra methyl group' at C-24 of the side chain of ergosterol (2) comes from methionine and Parks[5] proved that *S*-adenosylmethionine is the immediate methyl donor in this reaction.

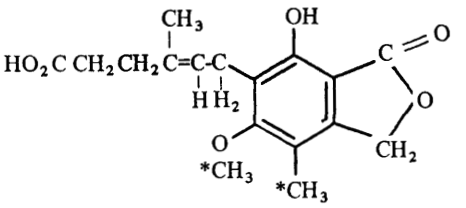
In recent years the subject of *C*-methylation has attracted widespread interest (for reviews, see refs. 6—10).

In the following we shall try to answer several questions concerning the detailed mechanisms of such *C*-alkylation reactions, which lead to branched-chain fatty acids, to various polycyclic aromatic and heterocyclic compounds, and to fungal and plant sterols.

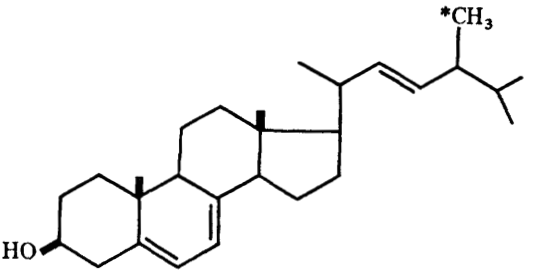
1 The Mechanism of *C*-Methylation *in vivo*

By use of [*Me*-$^2H_3$]methionine it was shown that in two particular cases, *i.e.* the biosynthesis of tuberculostearic acid (3) from oleic acid and the biosynthesis

* This Review covers the subject of the second R. A. Morton Lecture delivered on April 15th, 1969 at the Biochemistry Department, Liverpool.
† In all formulae carbon atoms carrying an asterisk are due to alkyl transfer from methionine.
[1] R. A. Morton, 'Biochemistry of Quinones', Academic Press, London and New York, 1965.
[2] A. J. Birch, D. Elliott, and A. R. Penfold, *Austral. J. Chem.*, 1954, **7**, 169.
[3] A. J. Birch, R. J. English, R. A. Massy-Westropp, M. Slaytor, and H. Smith, *Proc. Chem. Soc.*, 1957, 204.
[4] G. J. Alexander, A. N. Gold, and E. Schwenk, *J. Amer. Chem. Soc.*, 1957, **79**, 2967,
[5] L. W. Parks, *J. Amer. Chem. Soc.*, 1958, **80**, 2023.
[6] E. Lederer, *Biochem. J.*, 1964, **93**, 449.
[7] E. Lederer, *Experientia*, 1964, **20**, 473.
[8] E. Lederer, *Israel J. Med. Sci.*, 1965, **1**, 1129.
[9] R. B. Clayton, *Quart. Rev.*, 1965, **19**, 201.
[10] C. J. Sih and H. W. Whitlock, jun., *Ann. Rev. Biochem.*, 1968, **37**, 661.

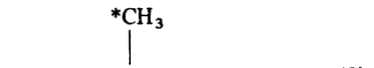


(1)



(2)

of ergosterol (2), only two of the hydrogen atoms of the methyl group of methionine are retained ('CD_2-mechanism')[11] whereas in the *C*-methylation of aromatic and heterocyclic as well as of some aliphatic compounds [*e.g.* the α-smegmamycolic acids (4)] all three hydrogen atoms of methionine are retained ('CD_3-mechanism').[12–15]



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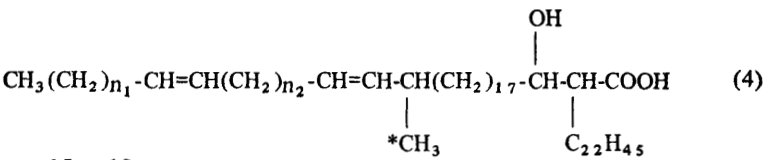


(4)

n_1 = 15 to 19

n_2 = 12 to 16

It was first thought that these two mechanisms differed radically in the transfer of either a CH_3 or only a CH_2 group from methionine; we shall see that this is not the case, the 'CD_2 mechanism' being due to the intermediary formation of a

[11] (*a*) G. Jauréguiberry, J. H. Law, J. A. McCloskey, and E. Lederer, *Compt. rend.*, 1964, **258**, 3587; (*b*) G. Jauréguiberry, J. H. Law, J. A. McCloskey, and E. Lederer, *Biochemistry*, 1965, **4**, 347.

[12] B. E. Tropp, J. H. Law, and H. M. Hayes, *Biochemistry*, 1964, **3**, 1837.

[13] G. Jauréguiberry, G. Farrugia-Fougerouse, H. Audier, and E. Lederer, *Compt. rend.*, 1964, **259**, 3108.

[14] G. Jauréguiberry, M. Lenfant, B. C. Das, and E. Lederer, *Tetrahedron*, 1966, Suppl. 8, part I, 27.

[15] L. M. Jackman, I. G. O'Brien, G. B. Cox, and F. Gibson, *Biochim. Biophys. Acta*, 1967, **141**, 1.

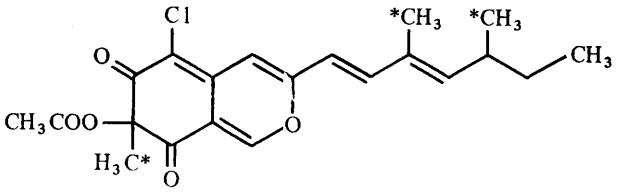
methylene derivative: a 24-methylene compound in the case of ergosterol[16–18] and 10-methylenestearic acid in the case of tuberculostearic acid.[19]

Two general cases of *C*-methylation may be distinguished:

A. *C*-Methylation of Double Bonds Activated by Electron-releasing Atoms (O or N).—This reaction of a strongly nucleophilic (for instance enolic) double bond can be represented as shown in Figure 1. It leads to an α-methylated ketone. If the enol is phenolic, the resulting product is a *C*-methylated phenol. This mechanism, already suggested by Birch[20,21] results in no loss of hydrogen from the methyl group and places the methyl on carbons often originally derived from the CH_2 of malonyl-CoA.

In agreement with this mechanism Tropp *et al.*[12] had found that the enzymatic conversion of uridine to thymine riboside in soluble RNA of *E. coli* proceeds with transfer of the whole CD_3 group of [*Me*-$^2H_3$]methionine.

We have shown that in compounds such as mycophenolic acid (1)[13] of *Penicillium brevi-compactum*, sclerotiorin (5) of *Penicillium slerotiorum*, the dihydromenaquinone-9 of *M. phlei* (6),[14] all three hydrogen atoms of the *C*-methyl group of [*Me*-$^2H_3$]methionine are retained in the final product, in agreement with the above mechanism. (The incorporation of a CD_3 group into menaquinones has been confirmed by Jackman *et al.*[15])



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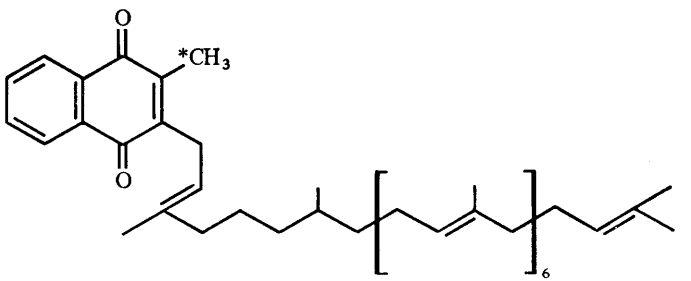
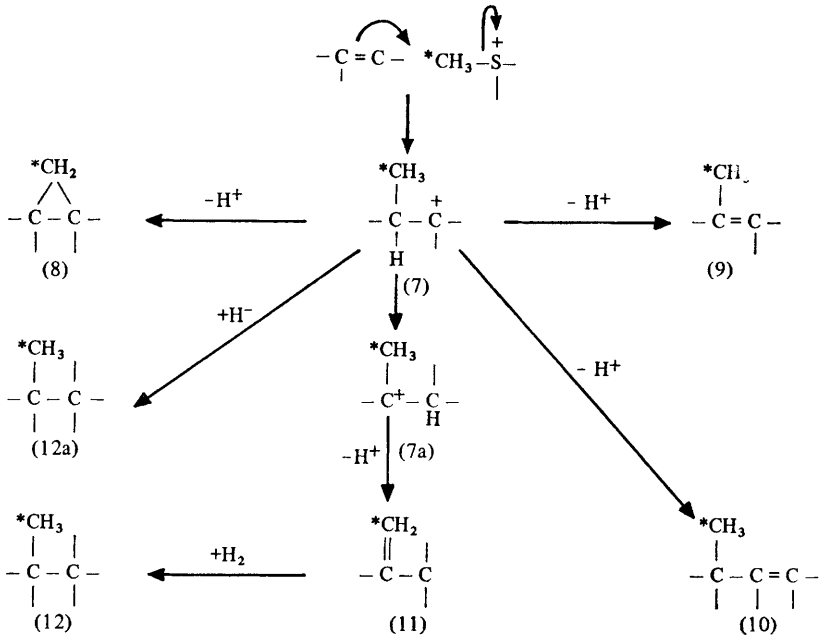


(6)

[16] D. H. R. Barton, D. M. Harrison, and G. P. Moss, *Chem. Comm.*, 1966, 595.
[17] M. Akhtar, M. A. Parvez, and P. F. Hunt, *Biochem. J.*, 1966, **100**, 38C.
[18] G. Goulston, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, 1967, **102**, 15C.
[19] G. Jauréguiberry, M. Lenfant, R. Toubiana, R. Azerad, and E. Lederer, *Chem. Comm.*, 1966, 855.
[20] A. J. Birch, *Progr. Chem. Org. Nat. Prod.*, 1957, **14**, 186.
[21] A. J. Birch, *Ann. Rev. Plant Physiol.*, 1968, **19**, 321.

B. *C*-Alkylation of Isolated Double Bonds.—Scheme 1 (modified from refs. 9 and 14) shows the different end products which can be formed through enzymatic alkylation of isolated double bonds by *S*-adenosylmethionine. It is considered that in all cases the whole methyl group is transferred and that an intermediate carbonium ion (7) is formed. The transfer of the whole methyl group to a non-activated aliphatic double bond has been proved in experiments on the biosynthesis of the α-smegmamycolic acids (4) by *Mycobacterium smegmatis* and of the methyl and ethyl side-chains of phytosterols by *Dictyostelium discoideum* (see below).



Scheme 1

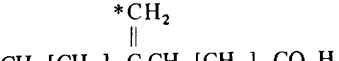
The carbonium ion (7) can be stabilised in different ways, to give the following structures:
1, a cyclopropane ring (8) (with loss of one H atom of the methyl group of methionine);[22] 2, a vinylic *C*-methyl group (9); 3, an allylic *C*-methyl group (10) [as in (4)]; 4, a methylene group (11) which after reduction leads to a *C*-methyl group (12) having only two of the hydrogen atoms of the methyl of methionine ('CD_2-mechanism');[11,16,17] 5, a methyl group (12a) having retained all three hydrogen atoms of the methyl of methionine.

The reaction (7)→(11)→(12) has been shown to lead from a 24-methylene

[22] S. Pohl, J. Law, and R. Ryhage, *Biochim. Biophys. Acta*, 1963, **70**, 583.

derivative of lanosterol to ergosterol;[16–18] it has been proved in our laboratory by the isolation of radioactive 10-methylenestearic acid (13) after incubation of *M. phlei* with [*Me*-^{14}C]methionine and by the conversion of the latter to [*Me*-^{14}C]-tuberculostearic acid (3).[19]

The hydride shift (7)→(7a) has been confirmed by Lenfant *et al.*:[23] after incubation of 9,10-dideuterio-oleic acid (14) with *M. phlei* cells dideuteriated tuberculostearic acid (14) was obtained; a detailed mass spectrometric analysis showed that the two deuterium atoms were on carbon-9.



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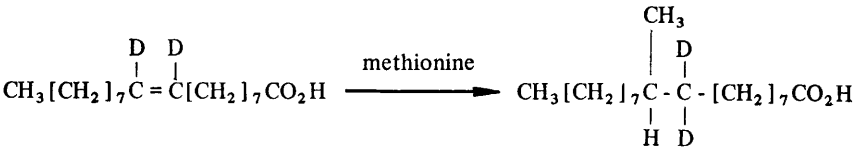
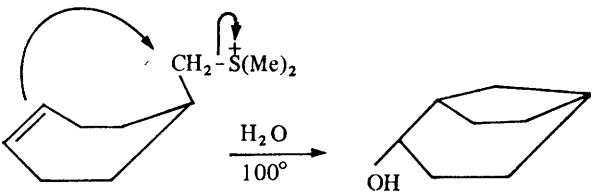


(14) (15)

In the steroid field analogous studies by Akhtar *et al.*,[24] Raab *et al.*,[25] and Goad and Goodwin[26] have shown that tritium at C-24 in the precursor is shifted to C-25 in the resulting sterol.

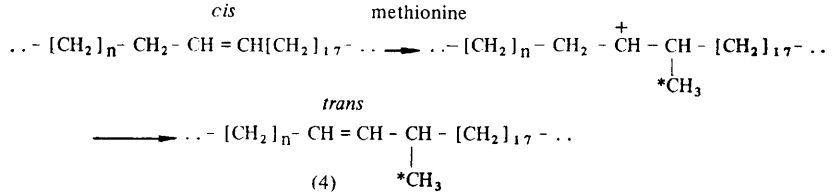
The step (7)→(10) of Scheme 1 may be illustrated by the α-smegmamycolic acids (4), where we have shown[14] that the lone methyl group with the allylic double bond retains all three deuterium atoms of [*Me*-^{14}C]methionine. Some non-methylated acids, possible precursors of α-smegmamycolic acid have been studied by Etémadi *et al.*[27]; they have the double bond in the expected position, predicted by the following sequence:



(16) (17)

[23] M. Lenfant, H. Audier, and E. Lederer, *Bull. Soc. chim. France*, 1966, 2775.
[24] M. Akhtar, P. F. Hunt, and M. A. Parvez, *Chem. Comm.*, 1966, 565; M. Akhtar, P. F. Hunt, and M. A. Parvez, *Biochem. J.*, 1967, **103**, 616.
[25] K. H. Raab, N. J. de Souza, and W. R. Nes, *Biochim. Biophys. Acta*, 1968, **152**, 742.
[26] L. J. Goad and T. W. Goodwin, *European J. Biochem.*, 1969, **7**, 502.
[27] A. H. Etémadi, F. Pinte and J. Markovits, *Compt. rend.*, 1966, **262**, 1343.

That non-activated (weakly nucleophilic) double bonds can be alkylated non-enzymically by a sulphonium grouping at a favourable distance was shown by Chuit and Felkin,[28] who obtained (17) by boiling (16) for 100 hr. in water.

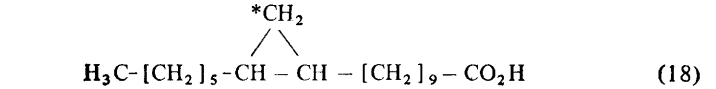


2 Can a Cyclopropane Derivative be a Precursor of a *C*-Methyl Compound?

Considering the quite analogous biogenetic origin of tuberculostearic acid (3) and lactobacillic acid (18), both produced by *C*-alkylation of a *cis* unsaturated acid by *S*-adenosylmethionine,[29,30] the question arises; can 9,10-methyleneoctadecanoic acid (dihydrosterculic acid) (13) formed from oleic acid, be a precursor of tuberculostearic acid (3) (see refs. 6—8)?

A similar question in the sterol field (opening of a cyclopropane ring to give a *C*-methyl group) has been raised several times in recent years and will be mentioned below.

The possibility of an opening of a cyclopropane ring to give a *C*-methyl compound had already been studied a few years ago in our laboratory, because we thought that such a conversion would give an obvious explanation of the 'CD_2' mechanism leading to tuberculostearic acid.[6–8] At that time, however, incubation of [^{14}C]-9,10-methylenestearic acid (19) with growing cells of *M. smegmatis* did not yield any labelled tuberculostearic acid (3).



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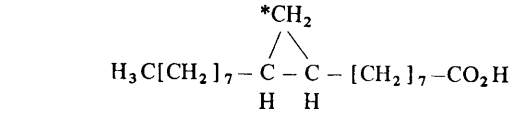
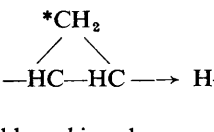


(19)

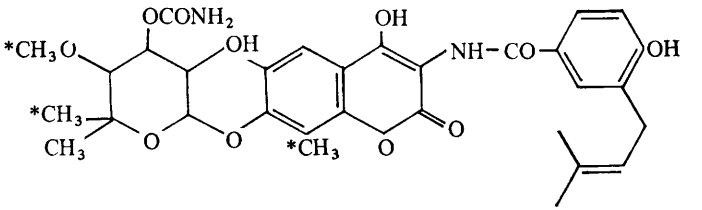
These experiments have been repeated recently with *M. phlei*,[31] but have not yet given clear-cut results; incorporation of radioactivity from [^{14}C]methylenedihydrosterculic acid (19) was very weak, less than 20% of the incorporation

[28] C. Chuit and H. Felkin, *Compt. rend.*, 1967, **264**, 1412.
[29] K. Hofmann and T. Y. Liu, *Biochim. Biophys. Acta*, 1960, **37**, 364.
[30] W. J. Lennarz, G. Scheuerbrandt, and K. Bloch, *J. Biol. Chem.*, 1962, **237**, 664.
[31] G. Jauréguiberry, D. Mercier, and E. Lederer, unpublished.

observed in parallel experiments with [^{14}C]-10-methylenestearic acid (13);[32] it can be concluded that, if the reaction  is possible in *M. phlei*, it proceeds with low yields and is only a secondary pathway.

3 *C*-Methylation of an Aliphatic Polyketide Precursor versus *C*-Methylation of an Aromatic Compound

Taguchi, Shibata, and Yamazaki[33,34] studying the biosynthesis of atranorin and usnic acid have found evidence that methylation precedes the aromatisation step. The same is true for the biosynthesis of the tetracyclines which has been extensively studied by McCormick[35] and there seems to be a general tendency to



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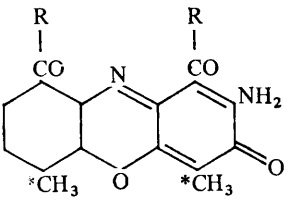

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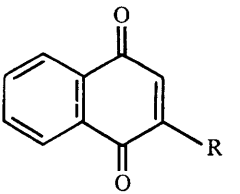


R = prenyl side chain

(22)

[32] R. Toubiana, G. Jauréguiberry, and E. Lederer, *Bull. Soc. chim. France*, 1967, 94.
[33] H. Taguchi, U. Sankawa, and S. Shibata, *Tetrahedron Letters*, 1966, 5211.
[34] M. Yamazaki and S. Shibata, *Chem. and Pharm. Bull. (Japan)*, 1966, **14**, 96.
[35] J. R. D.McCormick, in 'Biogenesis of Antibiotic Substances', ed. Z. Vanek and Z. Hostalek, Academic Press, New York, 1965, 73.

believe that in most cases the hypothetical polyketide precursor is methylated before cyclisation to the aromatic derivative. It is thus useful to enumerate the clear experimental pieces of evidence existing in some cases, where the *C*-methylation does operate on an aromatic ring. These are the following: the biosynthesis of novobiocine (20), where tyrosine is a precursor of the coumarin ring (Bunton *et al.*[36]); the biosynthesis of the actinomycins (21), where tryptophan is the precursor of the aromatic moiety (Sivak *et al.*[37]); the biosynthesis of the dihydromenaquinone-9 of *M. phlei* (6) studied by Azerad *et al.*[38] with cell-free extracts.

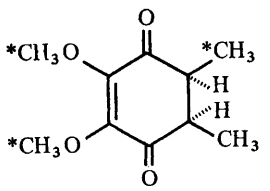
In these experiments, various demethylmenaquinones (22) incorporate the radioactivity of [*Me*-^{14}C]methionine.[39]

It seems worth mentioning that in nearly all these cases the *C*-methylation of the aromatic ring is operative in the *o*-position of a phenolic hydroxy-group. A review of the literature seems to show that no example is yet known of *C*-methylation of an aromatic ring not containing a phenolic hydroxy-function.

The following three sections will give some details of three cases of *C*-methylation which have been studied quite recently in more detail in our laboratory; two of these will give some insight into enzymatic mechanisms of *C*-alkylation reactions.

4 The Story of Gliorosein and Aurantiogliocladin

Gliorosein (23) is produced by the fungus *Gliocladium roseum* and oxidised in the culture medium to aurantiogliocladin (24); it has been shown that gliorosein is derived *via* the acetate plus malonate pathway (Bentley and Lavate,[40] Packter and Steward[41]). Birch *et al.*[42] have studied the incorporation of C_1 units and have found that, as expected, the *O*-methyl groups and one of the *C*-methyl groups is derived by *C*-methylation of a polyacetate precursor.



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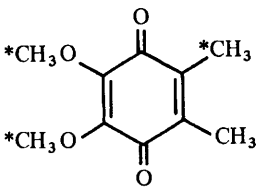

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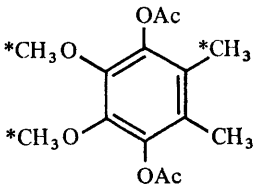


(25)

[36] C. A. Bunton, G. W. Kenner, M. J. T. Robinson, and B. R. Webster, *Tetrahedron*, 1963, **19**, 1001.
[37] A. Sivak, M. L. Meloni, F. Nobili, and E. Katz, *Biochim. Biophys. Acta*, 1962, **57**, 283.
[38] R. Azerad, M. O. Cyrot, and E. Lederer, *Biochem. Biophys. Res. Comm.*, 1967, **27**, 249.
[39] O. Samuel and R. Azerad, *F.E.B.S. Letters*, 1969, **2**, 336.
[40] R. Bentley and W. V. Lavate, *J. Biol. Chem.*, 1965, **240**, 532.
[41] N. M. Packter and M. W. Steward, *Biochem. J.*, 1967, **102**, 122.
[42] A. J. Birch, R. I. Fryer, and H. Smith, *Proc. Chem. Soc.*, 1958, 343.

More recently, Steward and Packter[43] have reported that only two of the H atoms of the methyl group of methionine are found in the *C*-methyl group of gliorosein (whereas three were found in the *O*-methyl groups). As these results

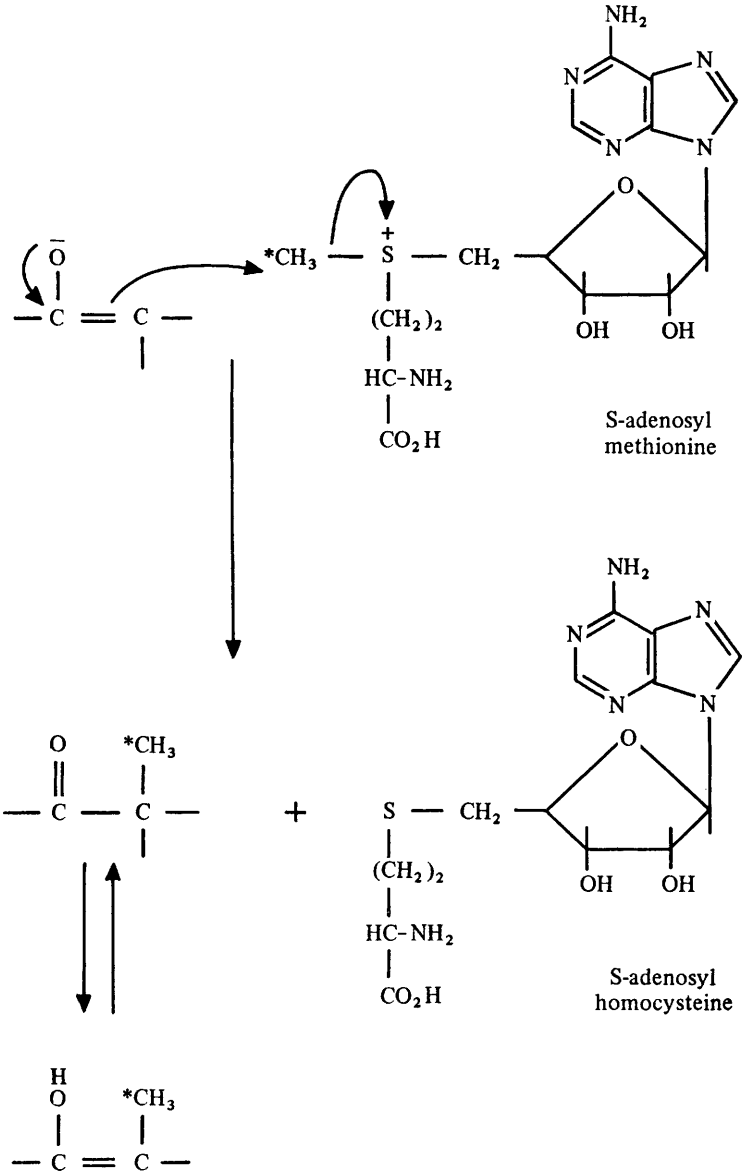


Figure 1

[43]M. W. Steward and N. M. Packter, *Biochem. J.*, 1968, **109**. 1.

seemed to contradict the mechanism suggested for methylation of ketones or phenols (see Figure 1), and as these authors had used doubly labelled [*Me*-^{3}H and *Me*-^{14}C]methionine, we thought it necessary to reinvestigate the biosynthesis of the metabolites of *Gliocladium roseum*, by use of [*Me*-$^{2}H_3$]methionine.

Mass spectrometry of (25) obtained by reductive acetylation of aurantioglio-cladin (24) isolated from a culture of the same strain as the one used by the above authors, showed unequivocally the incorporation of nine deuterium atoms, *i.e.*, the presence of three CD_3 groups (two *O*-Me and one *C*-Me). Ergosterol (2) isolated from the same culture was clearly $-CD_2$.[44]

This shows once more the danger of relying on results obtained with tritium-labelled molecules owing to the difficulty of evaluating the importance of various isotope effects (see also the critical comments of Sih and Whitlock[45]).

5 The Mechanism of the Biosynthesis of the Dihydromenaquinone-9 of *M. phlei*

Samuel and Azerad[39] have extensively studied the *C*-methylation of various demethylmenaquinones by cell-free extracts of *M. phlei*.

Various structural requirements for *C*-methylation could thus be defined:
(i) the presence of a fully aromatic naphthaquinone ring; partial or total saturation of the benzenoid ring inhibits *C*-methylation; benzoquinones are not methylated.
(ii) a *trans*-$\beta\gamma$-unsaturated side chain is necessary with at least two isoprenoid units. The best incorporation was obtained with a C_{15} and C_{20} side-chain, whereas the natural substrate has a C_{55} side-chain (Azerad *et al.*[38]).

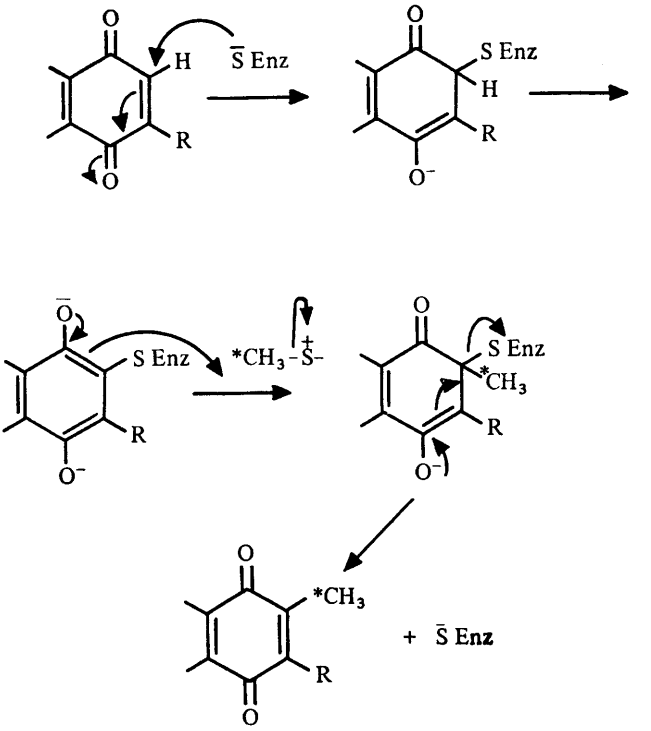
The rapid incorporation of radioactivity from [*Me*-^{14}C]methionine into the demethylmenaquinones, in a cell-free extract under aerobic conditions seemed, however, not to be in agreement with mechanistic considerations, which suggested that the substrate of the *C*-methylation reaction should be a phenol and not a quinone.

This dilemma could be obviated by assuming that the demethylmenaquinones react first with an SH group of the methylating enzyme, a reaction which is well known for analogous quinones (*e.g.* the plastoquinones, see Redfearn[46]).*†

Figure 2 shows the details of this hypothesis: addition of SH of the enzyme (SEnz) produces a ketone which then enolises and thus is a nucleophile, ready for *C*-methylation; after reaction with *S*-adenosylmethionine the SEnz group can be eliminated by the reversal of the addition reaction.

Figure 2 lends itself to experimental verification. Preliminary experiments of Azerad, Catala, and Lederer[48] have shown that, as expected, for this mechanism *p*-hydroxymercuribenzoate (10^{-3} M) inhibits strongly the methylation of the

* We are grateful to Dr. H. Felkin for helpful discussions concerning this mechanism.
† Quite recently, Whistance *et al.*[47] have used the ready addition of cystein to demethylmenaquinones for analytical purposes.
[44] M. Lenfant, G. Farrugia, and E. Lederer, *Compt. rend.*, 1969, **268**, *D*, 1986.
[45] C. J. Sih and H. W. Whitlock, *Ann. Rev. Biochem.*, 1968, **37**, 689.
[46] E. R. Redfearn, in 'Biochemistry of Quinones', ed. R. A. Morton, Academic Press, New York and London, 1965, 149.
[47] G. R. Whistance, J. F. Dillon, and D. R. Threlfall, *Biochem. J.*, 1969, **111**, 461.
[48] R. Azerad, F. Catala, and E. Lederer, unpublished data.



R = prenyl side chain

Figure 2

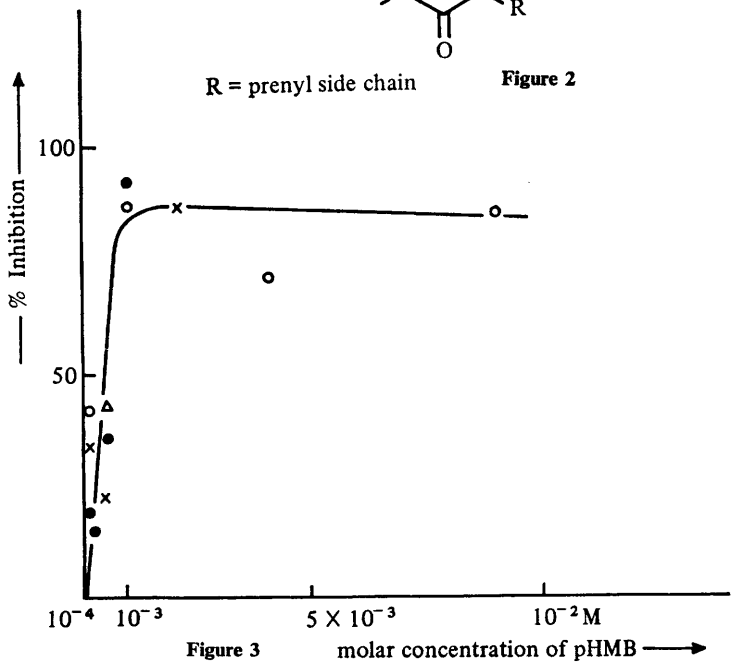


Figure 3

demethylmenaquinones (Figure 3); it seems significant that the biosynthesis of tuberculostearic acid, in the same extract is much less inhibited.

Figure 4 shows a hypothetical representation of the active sites of the *C*-methylase studied: site 1 recognises the naphthaquinone ring, site 2 binds the cofactor, *S*-adenosylmethionine, and has the reactive SH-group necessary for addition to C-2 of the demethylmenaquinones; site 3 has a hydrophobic character, for recognising the prenyl side chain.

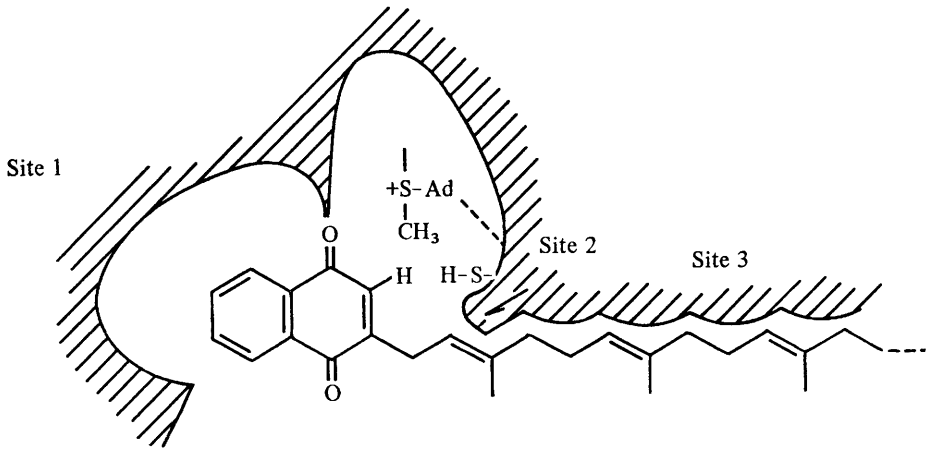


Figure 4

6 What is the 'Real' Substrate of the *C*-Methylation Reaction leading to Tuberculostearic Acid?

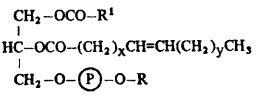
Zalkin *et al.*[49] and Chung and Law[50] have shown that in the *C*-alkylation leading from *cis*-vaccenic acid to lactobacillic acid (18) in *Clostridium butyricum* the methyl group of *S*-adenosyl methionine is transferred to the mono-unsaturated fatty acid esterified in a phosphatidylethanolamine. The substrate of the cyclopropane fatty acid synthetase is thus not free vaccenic acid, but a phosphatidylethanolamine containing the unsaturated acid (Figure 5).

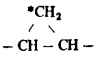
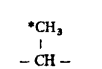
It was *a priori* probable that an analogous situation is true for the reaction oleic acid → tuberculostearic acid (3) performed by Mycobacteria. Yano *et al.*[51] studying the same reaction with *Nocardia polychromogenes*, also found that the radioactivity of [*Me*-^{14}C]methionine was incorporated into the phospholipids, but in this case phosphatidyl inositol showed the strongest labelling.

More recently, Akamatsu and Law,[52] using cell-free extracts of *M. phlei*, have reported experimental evidence for the following sequence:

[49] H. Zalkin, J. H. Law, and H. Goldfine, *J. Biol. Chem.*, 1963, **238**, 1242.
[50] A. E. Chung and J. H. Law, *Biochemistry*, 1964, **3**, 967.
[51] I. Yano, Y. Furukawa, and M. Kusunose, *J. Biochem.*, 1968, **63**, 133.
[52] Y. Akamatsu and J. H. Law, *Biochem. Biophys. Res. Comm.*, 1968, **33**, 172.

Figure 5 The substrate of the 'alkylases' producing lactobacillic and tuberculostearic acids



product	strain	R	references
	*Clostridium butyricum*	$-CH_2CH_2-NH_2$	Law et al. (49, 50)
	*Nocardia asteroides*	- inositol	Yano et al. (51)
	Mycobacterium phlei	- inositol oligomannosides $-CH_2CHOHCH_2O$ (P) diglyceride $-CH_2CH_2-NH_2$	Akamatsu and Law (52)
	Mycobacterium phlei	- inositol oligomannosides $-CH_2CHOHCH_2O$ (P) diglyceride	Azerad and El Hachimi (53)

Oleylphospholipid + *S*-adenosylmethionine → 10-methylene stearylphospholipid + *S*-adenosyl homocysteine

10-methylene stearylphospholipid + NADPH $\xrightarrow{+H^+}$ 10-methyl stearylphospholipid + NADP

They report that essentially all of the labelled tuberculostearic acid and 10-methylenestearic acid were components of phosphatidyl-inositol oligomannosides and phosphatidylethanolamine. They could never observe incorporation of label into a cyclopropane acid fraction.

In Orsay, Azerad, and El-Hachimi,[53] have been studying the nature of the 'real substrate' for some time and have obtained results which differ somewhat from those reported by Akamatsu and Law.[52] Most of the radioactivity of the phospholipid is incorporated into cardiolipins (5%) and phosphatidylinositolmannosides (20%); phosphatidylethanolamine was only very poorly labelled (*ca.* 5%), whereas 50% of the radioactivity of the whole phospholipid fraction was localised in a phosphorus-free compound migrating very similarly, but not identically to phosphatidylethanolamine in various chromatographic systems.

No free radioactive tuberculostearic acid was detected in these extracts. Incubation with [^{14}C]labelled tuberculostearic acid led only to a small incorporation in a fraction migrating as phosphatidylinositol, a pattern essentially different to that found when the extract is incubated with [*Me*-^{14}C]methionine. These experiments confirm that methylation occurs only on oleic acid esterified in a phospholipid molecule.

The *C*-alkylases acting on phospholipid-bound unsaturated acids are thus related to the *N*-methylase (of rat liver) described by Gibson *et al.*[54] which methylates phosphatidylethanolamine to phosphatidylcholine.

[53] R. Azerad and Z. El-Hachimi, unpublished data.
[54] K. D. Gibson, J. D. Wilson, and S. Udenfriend, *J. Biol. Chem.*, 1961, **236**, 673.

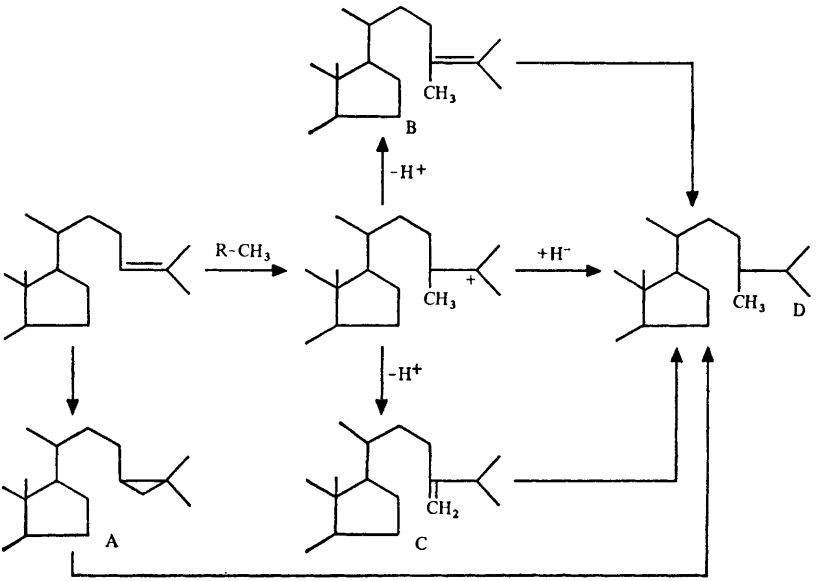
II: Problems Concerning Phytosterol Biosynthesis

1 The Mechanism of the First Alkylation Step leading to Ergosterol and to Precursors of the C-29 Phytosterols

Scheme 2 put forward by Frantz and Schroepfer[55] indicates the different pathways from a 24,25 unsaturated triterpene or sterol derivative to C-24 alkylated compounds.*

The following comments might be useful:

Compound A: no natural sterol or triterpene derivative having a 24,25-methylene bridge seems yet to have been encountered; in our laboratory, Toubiana has synthesised ^{14}C-24,25-methylenedihydrolanosterol (26)[57] and has incubated it with growing yeast cells. Ergosterol (2) isolated from this experiment was not radioactive, showing that reaction A→D is not possible under these conditions.[58]†



Scheme 2

* The necessity of the C-24 double bond for the introduction of the alkyl group at C-24 has been shown by Russell *et al.*[56]; neither 24,25-dihydrolanosterol nor cholesterol underwent alkylation.

† Under the same conditions 24-methylenedihydrolanosterol (30) is transformed into ergosterol (Barton *et al.*,[16] Akhtar *et al.*[17]).

[55] I. D. Frantz and G. J. Schroepfer, *Ann. Rev. Biochem.*, 1967, **36**, 691.

[56] P. T. Russell, R. T. van Aller, and W. R. Ness, *J. Biol. Chem.*, 1967, **242**, 5802.

[57] R. Toubiana and E. Lederer, *Bull. Soc. chim. France*, 1965, 2563.

[58] R. Toubiana, unpublished data.

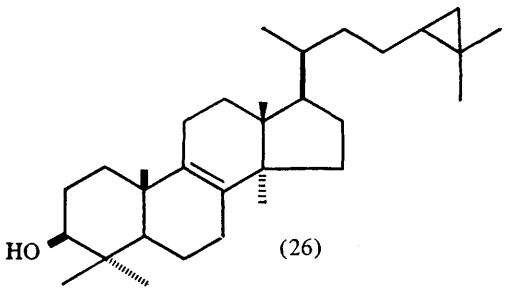
The opening of a cyclopropane ring leading to a *C*-methyl group has been considered by several authors studying phytosterol biosynthesis following the observations of Schreiber *et al.*,[59] Ourisson *et al.*,[60] and Goad and Goodwin,[61–63] that lanosterol (27) is absent in higher plants, whereas cycloartenol (28) was always found.

Pollinastanol (29) isolated by Barbier *et al.*[64,65] from pollen could be an intermediate between cycloartenol (28) and phytosterols.

Very recently, experimental evidence for the transformation of cycloartenol (28) to phytosterols has been announced by Ourisson *et al.*,[66] and by Hall *et al.*,[67] and also Devys *et al.*[68]

Compound B: no natural sterol or triterpene derivative having the side-chain of B seems to be known; an isomer, ascosterol, with a C-23,24 double bond is produced by yeast[69] and could be synthesised *via* compound B or C.

Compound C: the formation of a methylene derivative explains the loss of one of the H atoms of methionine observed by Jauréguiberry *et al.*[11] during ergosterol



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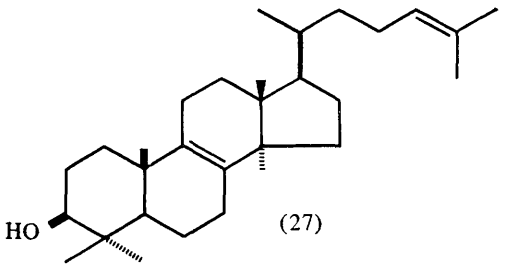
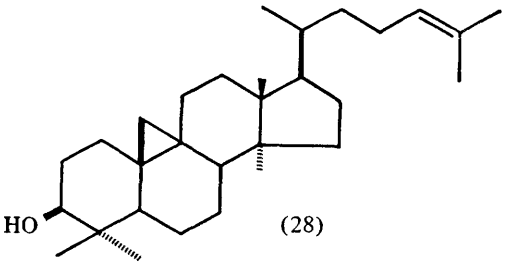


(27)

[59] M. von Ardenne, G. Osske, K. Schreiber, K. Steinfelder, and R. Tümmler, *Kulturpflanze*, 1965, **13**, 102, 115.
[60] P. Benveniste, L. Hirth, and G. Ourisson, *Phytochemistry*, 1966, **5**, 31, 45.
[61] L. J. Goad and T. W. Goodwin, *Biochem. J.*, 1966, **99**, 735.
[62] L. J. Goad, 'Terpenoids in Plants', ed. J. B. Pridham, Academic Press, London, 1967, 159.
[63] L. J. Goad, B. L. Williams, and T. W. Goodwin, *European J. Biochem.*, 1967, **3**, 232.
[64] M. F. Hügel, M. Barbier, and E. Lederer, *Bull. Soc. chim. France*, 1964, 2012.
[65] M. Devys and M. Barbier, *Bull. Soc. Chim. biol.*, 1967, **49**, 865.
[66] M. J. E. Hewlins, J. D. Ehrhardt, L. Hirth, and G. Ourisson, *European J. Biochem.*, 1969, **8**, 184.
[67] J. Hall, A. R. H. Smith, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, 1969, **112**, 129.
[68] M. Devys, A. Alcaide, and M. Barbier, *Bull. Soc. Chim. biol.*, 1969, **51**, 133.
[69] W. Fürst, *Annalen*, 1966, **699**, 206.



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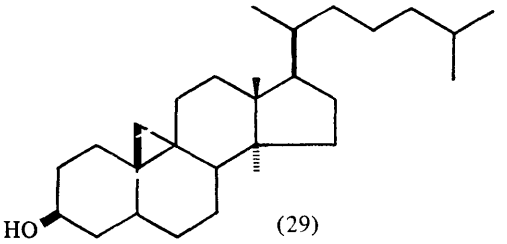
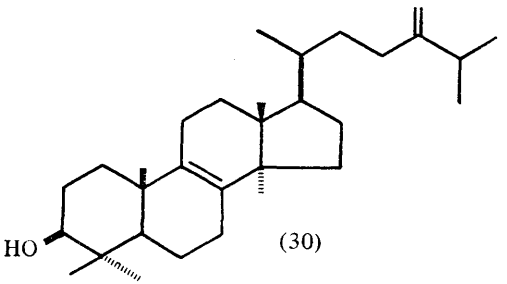
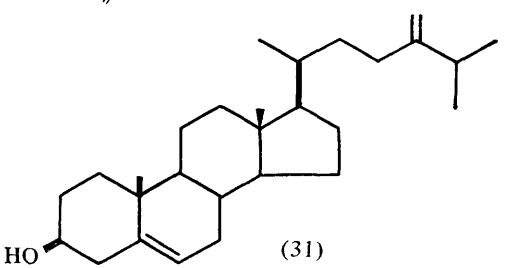


(29)

biosynthesis; the hydride shift occurring during the stabilisation of the intermediary carbonium ion has been studied by Akhtar *et al.*,[24] Raab *et al.*,[25] and Goad and Goodwin.[26] Many natural 24-methylene compounds have been isolated (see ref. 70). Some of these, such as euphorbol, cycloeucalenol, and the



(30)



(31)

[70] G. Ourisson, P. Crabbé, and O. Rodig, 'Tetracyclic Triterpenes', Hermann, Paris and Holden-Day, San Francisco, 1964.

eburicoic acid group seem to be 'end products', whereas others, in particular 24-methylenedihydrolanosterol (30), or 24-methylenecholesterol (31) may be intermediates of phytosterol biosynthesis.

Compound D can be formed from the intermediary carbonium ion by four different ways: two of these A→D and C→D lead to a methyl group having only two hydrogen atoms from methionine [as shown for ergosterol (2) in *Neurospora crassa*], whereas the others lead to a methyl group containing all three H atoms of methionine. Such a case has been observed by Lenfant *et al.*;[71] a minor fraction of the sterols of the slime mould *Dictyostelium discoideum* (a 4,24-dimethyl cholestanol) has three deuterium atoms in its 24-methyl group.

The reaction C→D has been proved by Alcaide *et al.*[72] who showed that 24-methylenecholesterol (31) gives a 24-methylcholesterol (probably campesterol) in tobacco leaves and by van Aller *et al.*[73] who found that the former was converted into 24-methylcholesterol by seeds of *Pinus pinea.*

2 At What Stage does the First *C*-Alkylation of the Triterpene or Sterol Side-chain Occur?

This is still a most controversial question and the best answer is probably: it can occur at any stage. Let us only consider the arguments in favour of the extreme possibilities: *C*-alkylation at the C_{30} level and at the C_{27} level.

A. *C*-Alkylation at the C_{30} (Lanosterol or Cycloartenol) Level.—This is of course well documented because a whole series of tetracyclic C-31 derivatives are known, which contain a C-24 methylene group (see ref. 70) or a methyl group (*e.g.* cyclolaudenol).

Goulston *et al.*[74] on the other hand, having isolated from *Phycomyces blakesleeanus* and from *Aspergillus fumigatus* lanosterol (27), 24-methylenedihydrolanosterol (30), and possibly 14-demethyl-24-methylenelanosterol, as well as four α-methyl-sterols containing a methylene group at C-24, conclude: 'we believe that transmethylation does occur at the lanosterol level to produce 24-methylenedihydrolanosterol, which is subsequently modified by loss of the 4α-, 4β-, and 14α-methyl groups and double-bond rearrangements to give ergosterol.'

The experiments of Akhtar *et al.*[17] and of Barton *et al.*[16] showing that 24-methylenedihydrolanosterol (30) can be metabolised to ergosterol (2) in yeast do not prove, however, that the natural sequence of reactions is:

$C_{30}\rightarrow C_{31}\rightarrow \rightarrow \rightarrow C_{28}$, but show only that the reaction $C_{31}\rightarrow \rightarrow \rightarrow C_{28}$

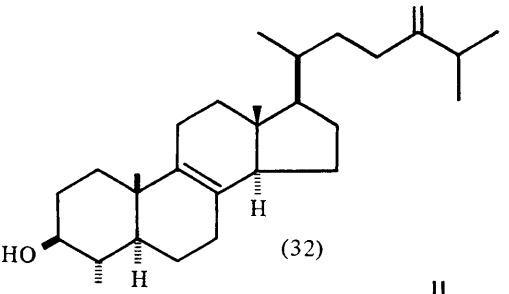
is possible *in vivo.*

[71] M. Lenfant and J. Varenne, unpublished data.
[72] A. Alcaide, M. Devys, J. Bottin, M. Fétizon, M. Barbier, and E. Lederer, *Phytochemistry* 1968, **7**, 1773.
[73] R. T. van Aller, H. Chikamatsu, N. J. de Souza, J. P. John, and W. R. Nes, *Biochim. Biophys. Acta*, in the press.
[74] G. Goulston, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, 1967, **102**, 15c.

Barton *et al.*[75] describing the isolation of 4α-methyl-24-methylene-24,25-dihydrozymosterol (32) from yeast and the incorporation of this new sterol, of 4α-methylzymosterol and of obtusifoliol (33) into ergosterol (respective yields 12, 15, and 8%) point out that these results show the 'possibilities of two or more biosynthetic pathways to ergosterol, or nonspecific enzyme systems which accept unnatural precursors'.

In a recent paper on the properties of a yeast sterol demethylase, Moore and Gaylor[76] conclude that 'side-chain methylation may accompany or precede complete demethylation of lanosterol'.

The lack of specificity of the alkylation enzymes is particularly well borne out by a paper by Russell *et al.*[56] where a cell-free extract of peas is shown to be able to alkylate lanosterol (27), cycloartenol (28) and desmosterol to the respective C-24 methylene derivatives.



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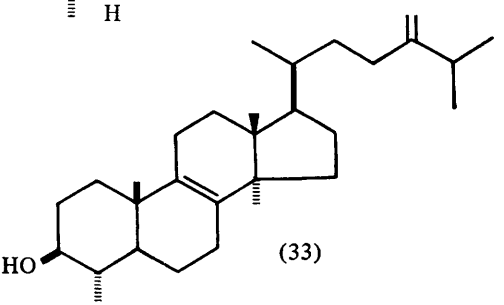


(33)

This reminds us of the apparent non-specificity of the saturation of the C-24–C-25 double bond of various precursors leading to cholesterol: Dempsey[77] has shown that this saturation can occur *in vivo* with zymosterol, or desmosterol, or cholesta-4,7,24-trienol.

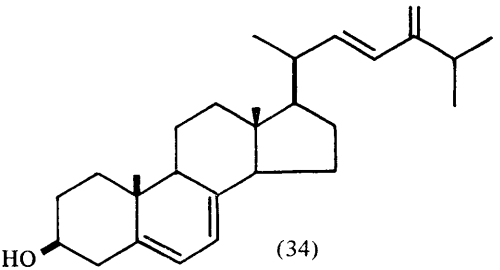
All these observations suggest that the enzymes 'manipulating' the side-chains of sterols are apparently not very 'critical' as concerns the tetracyclic structure and *vice versa*; the enzymes which dealkylate the C_{30} triterpenes at C-4 and those which 'move around' the tetrasubstituted double bond of lano-

[75] D. H. R. Barton, D. M. Harrison, and D. A. Widdowson, *Chem. Comm.*, 1968, 17.
[76] J. T. Moore and J. L. Gaylor, *Arch. Biochem. Biophys.*, 1968, **124**, 167.
[77] M. E. Dempsey, *J. Biol. Chem.*, 1965, **240**, 4176.

sterol and its analogues are apparently 'not interested' in the precise structure of the side-chain.

It is thus very difficult to get valid information as to the 'natural' pathway(s). They may vary depending on the category of organism or experimental conditions.

B. *C*-Alkylation of a C_{27} Precursor.—A survey of pollen sterols, where cholesterol, desmosterol, 24-methylenecholesterol (31), a C-24 methyl-sterol and ethylidene- and ethyl-sterols had been found, had led us to postulate that the *C*-alkylation at C-24 occurs mostly at the C_{27} level, *i.e.* with compounds like zymosterol, or desmosterol as precursors and 24-methylenecholesterol (31) as the first alkylation product (Hugel *et al.*[78]). More recently, Katsuki and Bloch[79] have shown that yeast cells convert zymosterol into ergosterol *via* an intermediate which they suppose to be ergosta-5,7,22,24-tetraene-3β-ol (34). Their experiments prove, at any rate, that in yeast the *C*-alkylation is possible *in vivo* with a C_{27} sterol as precursor.



(34)

Quite recently, Alcaide *et al.*[80] have obtained a radioactive 24-methylcholesterol (campesterol?) by incubating tobacco leaves with [3-^{3}H]desmosterol; in this experiment the C_{29} sterols were practically not labelled. This is again an indication that the first *C*-alkylation can occur at the C_{27} stage also in higher plants.

Here again, we might speculate that the methylating enzymes are not very specific for the structure of the tetracyclic portion of the molecule, so that they can 'operate' on different precursors.

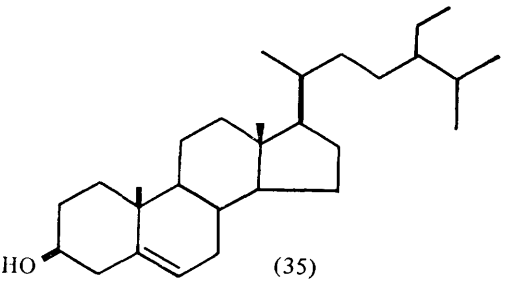
3 The Mechanism of the Second Alkylation Step Leading to the Ethyl or Ethylidene Side Chains of Phytosterols

A double *C*-methylation reaction producing the ethylidene and ethyl side-chains of the C-29 phytosterols had been postulated simultaneously by Birch[81] and Castle, Blondin, and Nes, in 1963,[82] and proved experimentally by the latter

[78] M. F. Hügel, W. Vetter, H. Audier, M. Barbier, and E. Lederer, *Phytochemistry*, 1964, **3**, 7.
[79] H. Katsuki and K. Bloch, *J. Biol. Chem.*, 1967, **242**, 222.
[80] A. Alcaide, M. Devys, and M. Barbier, *F.E.B.S. Letters*. 1969, **3**, 257.
[81] A. J. Birch, in 'Chemical Plant Taxonomy', ed. T. Swain, Academic Press, New York, 1963, 163.
[82] M. Castle, G. A. Blondin, and W. R. Nes, *J. Amer. Chem. Soc.*, 1963, **83**, 3306.

authors for β-sitosterol (35), by Bader *et al.*[83] for spinasterol (36) and by Barbier *et al.* for fucosterol (37).[84] The three groups showed that the radioactivity of [*Me*-^{14}C]methionine was incorporated into the ethyl or ethylidene side-chains of phytosterols and that both carbon atoms (C-28 and C-29) were equally radioactive.

Scheme 3, suggested by Lenfant *et al.*[85] (a modified version of the scheme of



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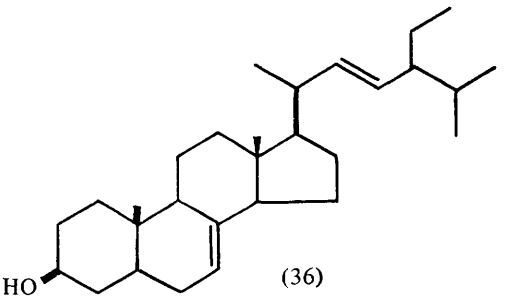

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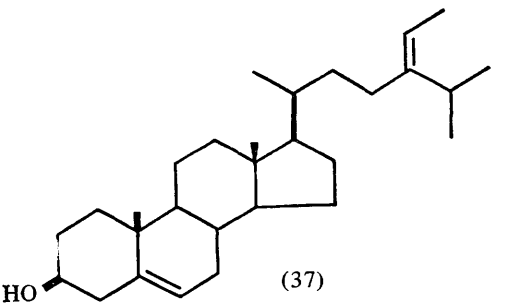
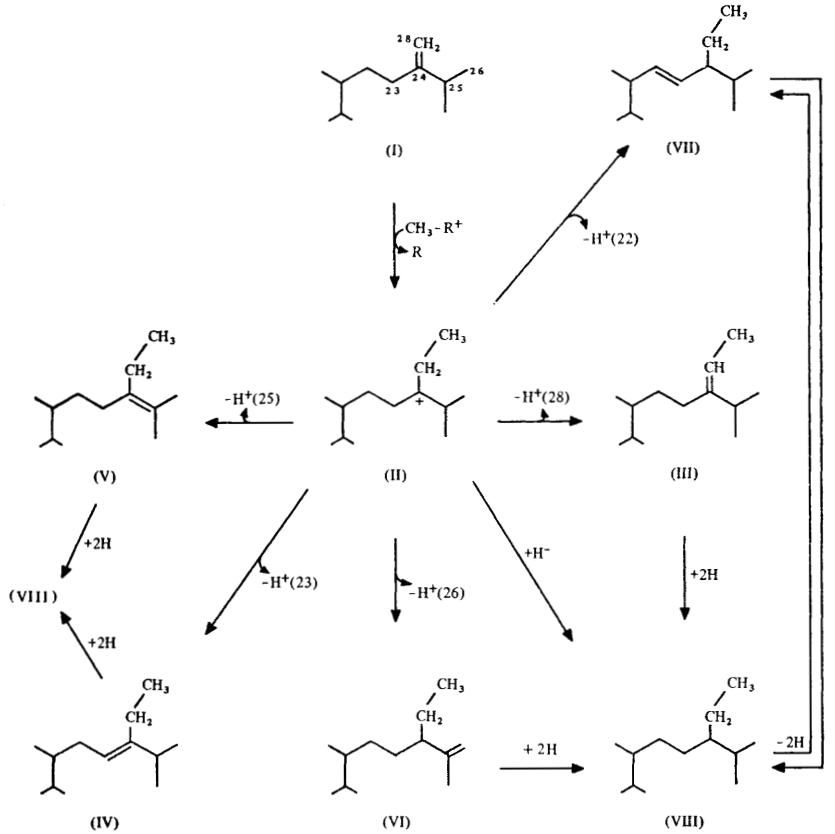


(37)

[83] S. Bader, L. Guglielmetti, and D. Arigoni, *Proc. Chem. Soc.*, 1964, 16.
[84] V. R. Villanueva, M. Barbier, and E. Lederer, *Bull. Soc. chim. France*, 1964, 1423; V. R. Villanueva, M. Barbier, and E. Lederer, 'Beitrage zur Biochemie und Physiologie von Naturstoffen', K. Mothes Festschrift, VEB G. Fischer Verlag, Jena, 1965, p. 509.
[85] M. Lenfant, R. Ellouz, B. C. Das, E. Zissmann, and E. Lederer, *European J. Biochem.*, 1969, **7**, 159.



Scheme 3

Smith *et al.*[86]) illustrates several possible pathways leading from a C-24 methylene compound to phytosterols with ethylidene and ethyl side-chains.

Castle *et al.*[82] had already put forward an analogous scheme in 1963, in which the carbonium ion (II) formed after the second methylation step can either lead to an ethylidene derivative (III) containing four, or to an ethyl derivative (VIII) containing five, hydrogen atoms derived from methionine.

In several biogenetic schemes (*e.g.* ref. 87) ethylidene derivatives (III) such as fucosterol (37), have generally been considered to be precursors of the ethyl derivatives (VIII), by analogy with methyl derivatives which have been shown to originate by saturation of intermediate methylene derivatives.

Goad *et al.*[87] had studied the biosynthesis of the phytosterol side-chain by use of [*Me*-^{14}C,^{3}H]methionine; they concluded that the ethyl side-chain [of

[86] A. R. H. Smith, L. J. Goad, T. W. Goodwin, and E. Lederer, *Biochem. J.*, 1967, **104**, 56c.
[87] L. J. Goad, A. S. A. Hammam, A. Dennis, and T. W. Goodwin, *Nature*, 1966, **210**, 1322.

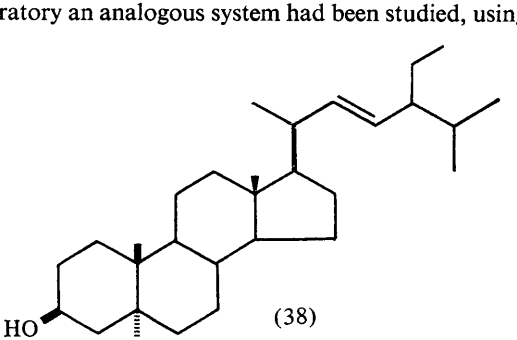
sitosterol (35) of maize and larch leaves] contains only four hydrogen atoms derived from methionine, thus indicating the sequence (I)→(II)→(III)→(VIII).

We thought that the same problem should be studied using [*Me*-$^2H_3$]methionine. The only difficulty was to choose a biological system permitting a high enough incorporation of the labelled methionine.

In the course of a discussion of this problem, Professor D. Arigoni suggested the use of the slime mould *Dictyostelium discoideum*, the sterols of which had been studied by Heftmann *et al.*[88] and Johnson *et al.*[89] As this organism feeds on *E. coli*, Dr. M. I. Krichevsky made the excellent suggestion that we should first grow a methionine-less mutant of *E. coli* on [*Me*-$^2H_3$]methionine and then feed these cells to the slime mould.

By use of this 'trick' a good incorporation of [*Me*-$^2H_3$]methionine was obtained;[85,90] mass spectrometry of the isolated sterol (stigmast-22-en-3β-ol) (38) showed without any doubt that five deuterium atoms had been incorporated into the ethyl side-chain,* excluding an ethylidene derivative as intermediate and indicating that reaction (I)→(VIII) proceeds (in this case) either by reduction of (II) by a hydride ion, or by elimination of H^+ [not *via* (III)] leading to other unsaturated compounds which could then be reduced.

Can this 'C_2D_5' mechanism be generalised? Certainly not. In Professor Goodwin's laboratory an analogous system had been studied, using the flagellate



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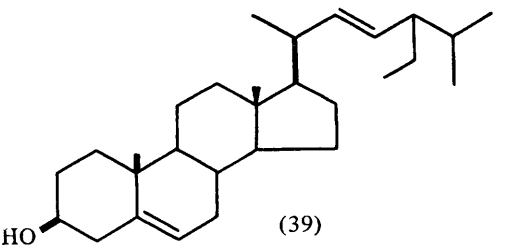


(39)

[88] E. Heftmann, B. E. Wright, and C. U. Liddel, *Arch. Biochem. Biophys.*, 1960, **91**, 266.
[89] D. F. Johnson, B. E. Wright, and E. Heftmann, *Arch. Biochem. Biophys.*, 1962, **97**, 232.
[90] M. Lenfant, E. Zissmann, and E. Lederer, *Tetrahedron Letters*, 1967, 1049.
* The side chain of the penta-deuteriated sterol (38) was isolated as *p*-phenylphenylacetate of α-ethyl-β-methylbutyric acid. A detailed mass spectrometric study confirmed that all five deuterium atoms are in the ethyl group (Lenfant *et al.*[85]).

Ochromonas malhamensis; in this case it was shown by a collaboration of the two groups[86] that only four deuterium atoms are incorporated into the side-chain of poriferasterol (39), thus suggesting that in this case fucosterol (37) can be an intermediate, in agreement with the experiments of Goad *et al.*[87]

Recently, Lenfant *et al.* (unpublished) have found that the slime mould *Physarum polycephalum* incorporates five D atoms of [*Me*-$^2H_3$]methionine into the ethyl side chain of its sterols (most probably stigmastanol, β-sitosterol, stigmasterol), whereas *Ochromonas danica* follows its cousin *O. malhamensis* by incorporating only four D atoms (into poriferasterol).

We thus have two cases for each of these 'C_2D_5' and 'C_2D_4' mechanisms; it seems that there is no relation between the mechanism of biosynthesis and the stereochemistry of the ethyl or methyl side-chain (Lenfant unpublished work).

Quite recently, van Aller *et al.*[74] have shown that in *Pinus pinea* seeds radioactive 28-isofucosterol yields radioactive β-sitosterol (35), thus confirming that, as expected, 24-ethylidene-sterols can be precursors of 24-ethyl-sterols.

4 At What Stage is the C-22–C-23 Double Bond Introduced?

Following the work on the 'C_2D_5' and 'C_2D_4' mechanisms described above, several possible schemes can be envisaged, involving the alkylation of a C-24–C-25 double bond with or without concomitant formation of the C-22–C-23 double bond frequently found in C_{28} and C_{29} sterols.

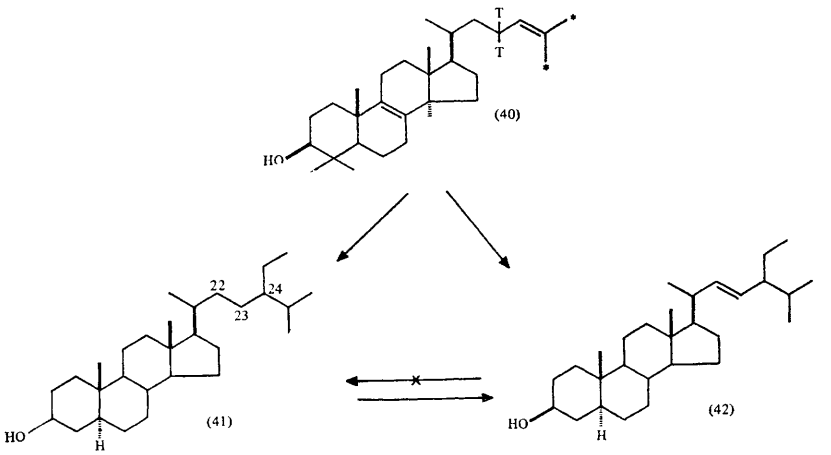
Recent experimental evidence indicates, however, that the C-22–C-23 double bond is introduced after the alkylation step by a more or less independent operation of desaturation of a saturated side-chain.

Johnson *et al.*[91] had studied the incorporation of [2-^{14}C]mevalonic acid into the phytosterols of *Solanum tuberosum* and found that the labelling of β-sitosterol started sooner and was more important than that of the Δ^{22} unsaturated stigmasterol. The latter was even absent after a 1 day growth period, suggesting that β-sitosterol is the precursor of stigmasterol (see also Kemp *et al.*[92]).

Recent experiments of Akhtar *et al.*[93] 'prove unambiguously that the enzyme participating in the introduction of the C-22–C-23 double bond of ergosterol can function without requiring the activation of an adjacent C-24–C-28 double bond'; these authors have shown that 24-methyldihydrolanosterol is converted into ergosterol in good yield (without prior conversion into a 24-methylene derivative), thus proving that the C-22–C-23 double bond can be introduced after *C*-alkylation at C-24.

Ellouz and Lenfant[94] have reported quite recently the results of experiments of incubation of a mixture of [26,27-$^{14}C_2$]lanosterol, and [23-^{3}H]lanosterol (40) with *Dityostelium discoideum*; both the saturated sterol (41) and the side-chain-

[91] D. F. Johnson, E. Heftmann, and G. V. C. Houghland, *Arch. Biochem. Biophys.*, 1964, **104**, 102.
[92] R. J. Kemp, L. J. Goad, and E. I. Mercer, *Phytochemistry*, 1967, **6**, 1609.
[93] M. Akhtar, M. A. Parvez, and P. F. Hunt, *Biochem. J.*, 1968, **106**, 623.
[94] R. Ellouz and M. Lenfant, *Tetrahedron Letters*, 1969, 609.



unsaturated sterol (42) were radioactive; the saturated sterol (41) had the same ratio T:^{14}C (4·12 ± 0·21) as the lanosterol mixture (4·36 ± 0·1), whereas the unsaturated sterol had a ratio of 3·32 ± 0·14. This shows that the double *C*-methylation of the lanosterol side-chain leads directly to the saturated sterol and that the side-chain double bond is introduced by desaturation of the saturated, already alkylated, side-chain. A further argument for this sequence is the fact that the specific radioactivity of the saturated sterol is higher than that of the unsaturated. Moreover, the same authors[95] have found that incubation of [3α-^{3}H]stigmastanol (41) with a growing culture of *D. discoideum* gives the radioactive C-22 unsaturated sterol (42) in a yield of 4·9%.

Patterson and Karlander[96] have recently reported an interesting observation: in *Chlorella ellipsoidea* ^{14}C-labelled fucosterol (37) was converted into clionasterol (with a saturated 24β-side-chain) and not into the C-22 unsaturated poriferasterol (39); this rules out the desaturation of clionasterol to poriferasterol. It thus seems that in this organism the C-22 double bond is introduced at an earlier stage (perhaps during stabilisation of the C-29 carbonium ion).

5 At what Stage does the Second Alkylation Step Occur?

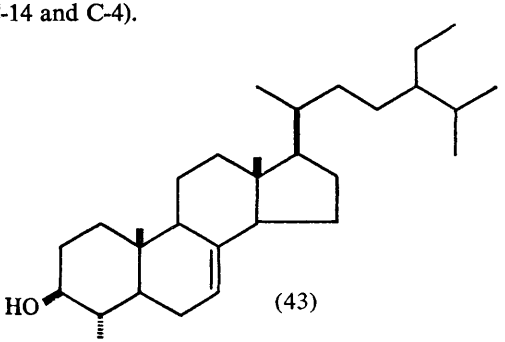
Until quite recently no C_{32} triterpene was known (*i.e.* a C_{30} triterpene with a C_2 side-chain at C-24). The second *C*-alkylation step thus certainly occurs after partial dealkylation (at C-14 or C-4).

Arthur and Loo[97] have isolated in 1967 the 'first C_{32} triterpene' from leaves of a *Lauraceae* (*Neolitsea pulchella*); its structure is not yet known.

At present only one 4-α-methyl compound with a C_2 side-chain at C-24

[95] M. Lenfant and R. Ellouz, unpublished data.
[96] G. W. Patterson and E. P. Karlander, *Plant Physiol.*, 1967, **42**, 1651.
[97] H. R. Arthur and S. N. Loo, *Tetrahedron Letters*, 1967, 3767.

seems to be known: citrostadienol (=ethylidenelophenol) (43); it thus seems that the second alkylation step does only very rarely occur before complete dealkylation (at C-14 and C-4).



(43)

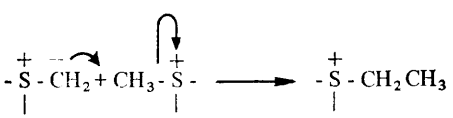
In our laboratory, Devys *et al.*[98] have shown that spinach leaves can transform 24-methylene-[23,25-^{3}H]dihydrolanosterol (30) to α-spinasterol (36), but it seems rather probable that the second alkylation step had taken place after partial dealkylation of the labelled precursor.

In tobacco leaves β-sitosterol is biosynthesised from 24-methylenedihydrolanosterol (30), whereas 24-methylenecholesterol (31) is only reduced to campesterol; in this case it is thus shown that the second alkylation step cannot be performed at the C_{28} level (Alcaide *et al.*[99]).

6 Is a *C*-Ethylation Reaction by Ethionine Possible?

In 1961 Fisher and Mallette[100] described the isolation, in small quantities, of a sulphur-containing amino-acid fraction from *E. coli* B and some other bacteria, which they considered to be ethionine (see also Loersch and Mallette[101]); they reported that ethionine was synthesised by wresting *E. coli* cells from radioactive sulphate and from radioactive methionine.

Knowing that double *C*-methylation can occur in nature one could have speculated that ethionine could be synthesised *in vivo* by 'auto-*C*-methylation' from two molecules of methionine. An ylide $-\overset{+}{S}-\overset{-}{C}H_2$ formed from one molecule of *S*-adenosylmethionine could react with a second molecule of *S*-adenosylmethionine to give *S*-adenosylethionine:



[98] M. Devys, A. Alcaide, M. Barbier, and E. Lederer, *Phytochemistry*, 1968, **7**, 7090.
[99] A. Alcaide, M. Devys, J. Bottin, M. Fétizon, M. Barbier, and E. Lederer, *Phytochemistry*, 1968, **7**, 1773.
[100] J. F. Fischer and M. F. Mallette, *J. Gen. Physiol.*, 1961, **45**, 1.
[101] J. D. Loersch and M. F. Mallette, *Arch. Biochem. Biophys.*, 1963, **103**, 272.

The possible transfer of the ethyl group to a C-24–C-25 unsaturated steroid precursor, *i.e.* a *C*-ethylation reaction must be considered, especially as it is known that free ethionine is easily 'activated' *in vivo* to *S*-adenosylethionine. Moreover, in some cases the addition of ethionine to culture media of micro-organisms has led to: *N*-ethylation (Stekol and Weiss;[102] Dulaney *et al.*;[103] Kaye *et al.*;[104] Rosen[105]), to *O*-ethylation (Jackson *et al.*[106]), or to *S*-ethylation (Parks;[107] Patterson *et al.*;[108] Argoudelis and Mason[109]).

Despite all the arguments in favour of the possibility of *C*-ethylation by ethionine, the answer to question 6 is negative. Firstly, we have shown with Villanueva and Barbier[110] that the fraction described by Ficher and Mallette[100] does not contain ethionine. This compound is thus to be deleted from the list of actually known natural amino-acids. Secondly, because several experiments with yeast (Spence *et al.* [111]), or with a cell-free pea extract (Castle *et al.*[112]) or with a methionine-less strain of *Neurospora crassa* supplied with a minimum amount of methionine and an excess of ethionine (Lenfant[113]) have failed to show any incorporation of radioactivity of the ethyl group of ethionine into the sterol fractions. Moreover, ethionine has been shown to inhibit *C*-methylation during tropolone biosynthesis (Bentley and Zwitkowits[114]).

We conclude that *C*-ethylation by ethionine does not seem to be possible.

7 How could one explain the 'CD_1' Problem and Complete Loss of Hydrogen Atoms of the Methyl Group of Methionine?

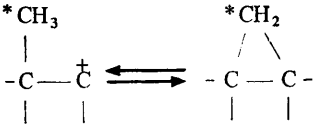
We have already seen that by using [*Me*-$^2H_3$]methionine one can obtain various *C*-methylated metabolites, some of which have a CD_3 group whereas others [tuberculostearic acid (3) and ergosterol (2)] have a CD_2H group.

In our first paper on the biosynthesis of these two compounds[11b] it was stated that a small percentage (7—13 %) of CD_1H_2 peaks were also present (see Table 1).

Are these 'CD_1' peaks due to a random loss of the H atoms of the methyl group of methionine (due to metabolic turnover), or to a more specific biochemical mechanism?

[102] J. A. Stekol and K. Weiss, *J. Biol. Chem.*, 1950, **185**, 577.
[103] E. L. Dulaney, I. Putter, D. Drescher, L. Chaiet, W. J. Miller, F. J. Wolf, and D. Hendlin, *Biochem. Biophys. Acta*, 1962, **60**, 447.
[104] A. M. Kaye, B. Fridlender, R. Salomon, and S. Bar-Meir, *Biochem. Biophys. Acta*, 1967, **142**, 1331.
[105] L. Rosen, *Biochem. Biophys. Res. Comm.*, 1968, **33**, 546.
[106] M. Jackson, E. L. Dulaney, I. Putter, H. M. Shafer, F. J. Wolf, and H. B. Woodruff, *Biochem. Biophys. Acta*, 1962, **62**, 616.
[107] L. W. Parks, *J. Biol. Chem.*, 1958, **232**, 169.
[108] E. L. Patterson, J. H. Hash, M. Lincks, P. A. Miller, and N. Bohonos, *Science*, 1964, **146**, 1691.
[109] A. D. Argoudelis and D. J. Mason, *Biochemistry*, 1965, **4**, 704.
[110] V. R. Villanueva, M. Barbier, C. Gros, and E. Lederer, *Biochem. Biophys. Acta*, 1966, **130**, 329.
[111] K. D. Spence, L. W. Parks, and S. K. Shapiro, *J. Bacteriol.*, 1967, **94**, 1531.
[112] M. Castle, G. A. Blondin, and W. R. Nes, *J. Biol. Chem.*, 1967, **242**, 5796.
[113] M. Lenfant, unpublished data.
[114] R. Bentley and P. M. Zwitkowits, *J. Amer. Chem. Soc.*, 1967, **89**, 676.

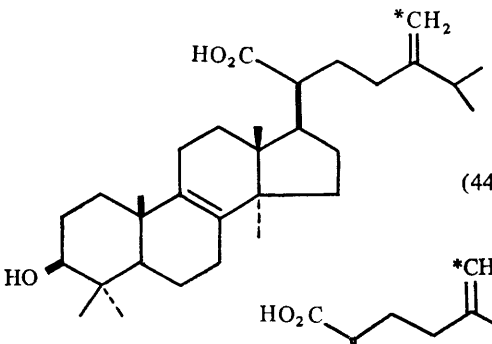
First we thought that the reversibility of the reaction



(suggested to us by Professor P. De Mayo), which would lead to a successive exchange of the original hydrogen atoms of the methyl group could explain the presence of these 'CD_1' peaks.

More recently, however, Lenfant[113] has found a more plausible explanation. By studying the mass spectra of deuteriated aurantiogliocladin (24) and of ergosterol (2), both obtained from the same culture of *Gliocladium roseum*, she found that there were no 'CD_1' and $< 3\,\%$ of 'CD_2' species in aurantiogliocladin, whereas in ergosterol the 'CD_1' species represents $20\,\%$ of the 'CD_2' species (Table 1). In mycophenolic acid (1) (another 'CD_3' case) there are no 'CD_1' peaks either; this shows that the formation of CD_1-ergosterol is not due to a random loss of deuterium atoms of the added [*Me*-$^2H_3$]methionine, but is more or less inherent in the 'CD_2' mechanism.

A closer inspection of the mass spectra of [*methylene*-$^2H_2$]eburicoic acid (44)[115] and of [*methylene*-$^2H_2$]pachymic acid (45)[116] revealed here too the absence of any significant 'CD_2' peak; this seems to rule out the loss of deuterium *via* a cyclopropane derivative.



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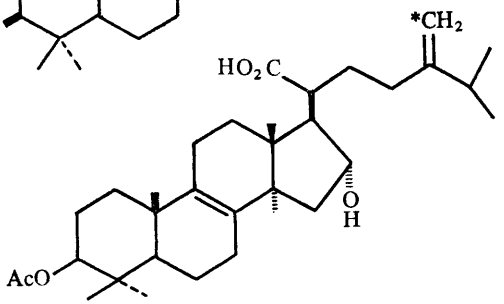

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[115] V. R. Villanueva, M. Barbier, and E. Lederer, *Bull. Soc. Chim. biol.*, 1967, **49**, 389.
[116] J. Polonsky and M. N. Ricroch, unpublished data.

Table *Molecular species formed by* C-*methylation with* [Me-$^2H_3$]*methionine* (*Lenfant, ref.* 113)

C-Methylated compounds	*Relative intensities of molecular ion (%)*										*Intensity of M+1 peaks in % of intensity of M+2 peaks*
	M	*M*+1	*M*+2	*M*+3	*M*+4	*M*+5	*M*+6	*M*+7	*M*+8	*M*+9	
Tuberculostearic acid (*Mycobacterium smegmatis*)	38	7	55								13
Ergosterol (*Neurospora crassa*)	16	13	71								18
Ergosterol (*Gliocladium roseum*)	15	14·5	70·5								20
Ergosterol (*Ochromonas danica*)	75	3·7	20·4								18
Eburicoic acid (*Polyporus sulphureus*)	20	2	78								2
Pachymic acid (*Daedalea quercina*)	62	<1	38								2
Aurantiogliocladin (*Gliocladium roseum*)	10·5	<1	<1	14	<1	<1	19·5	<1	2	53	
Mycophenolic acid (*Penicillium brevi-compactum*)	50			33			17				

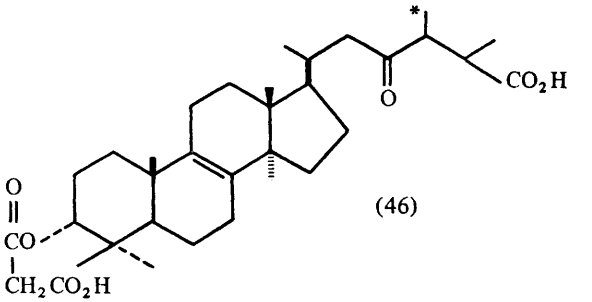
The 'CD_1' peaks thus seem to be present in significant amounts only in *C*-methyl compounds formed *in vivo* by reduction of a methylene compound [*e.g.* tuberculostearic acid (3) and ergosterol (2)]. Lenfant concludes that one plausible explanation is the reversibility of the reaction

$$=^{*}CH_2 \underset{-H_2}{\overset{+H_2}{\rightleftharpoons}} -^{*}CH_3$$

This enzymic desaturation* would not only explain the formation of CD_1 compounds, but also the complete loss of deuterium.

The presence of unlabelled ergosterol molecules in a methionine-less strain of *Neurospora crassa* grown in the presence of 100% pure [*Me*-$^2H_3$]methionine (see the Table) could be due to such a total loss.

Another, different, case of total loss of H atoms of the methyl group of methionine has been recently discovered by Polonsky and Ricroch[116] who have found that after incubation of the fungus *Daedalea quercinea* with [*Me*-$^2H_3$]-methionine, pachymic acid (45) incorporates two deuterium atoms, as expected, whereas carboxyacetylquercinic acid (46) does not contain any deuterium at all. Both compounds incorporated the radioactivity of [*Me*-^{14}C]methionine [31% in the case of (45) and 8% in the case of (46)]. Apparently the hydrogen atoms of the methyl group of (26) have been exchanged during the biosynthesis, owing to the peculiar structure of the side-chain.



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I wish to thank Dr. B. Tchoubar and Dr. H. Felkin for many enlightening discussions and Dr. R. Azerad, Dr. M. Barbier, Dr. J. Barnett, Dr. M. Lenfant, and Dr. J. Polonsky for reading the manuscript and for their helpful comments and criticism.

* This dehydrogenation reaction has an analogy in insect metabolism: Ritter and Wientjens[117] have shown that *Blattella germanica* dehydrogenates dihydrobrassicasterol to 24-methylene-cholesterol and that *Eurycotis floridana* dehydrogenates sitosterol to fucosterol; the unsaturated compounds are finally transformed to desmosterol and to cholesterol.

Recent experiments of Alais and Barbier[118] in Gif seem to confirm these results, showing that *Locusta migratoria* produces fucosterol, 24-methylenecholesterol, and desmosterol from [3-^{3}H]sitosterol.

[117] F. J. Ritter and W. H. J. M. Wientjens, *TNO-Nieuws*, 1967, 381.

[118] J. P. Alais and M. Barbier, unpublished data.