THE BIOSYNTHESIS OF CYCLOPROPANE FATTY ACIDS

By

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ABSTRACT

Mechanistic information has been obtained for biological cyclopropane fatty acid formation by L. plantarum. Deuterium labelling studies showed that when a methylene bridge is constructed across the double bond of an olefinic fatty acid, no scrambling or loss of label occurs at the vinylic or the allylic position of the olefin. One of the consequences of these results is that any mechanism whereby the olefin is activated at the allylic position has been conclusively ruled out.

Up to 18% of a d_1-cyclopropyl fatty acid was formed in feedings using methionine-methyl-d_3. It was shown that exchange had not occurred at the methionine stage. Exchange of a protonated cyclopropane intermediate is postulated to account for the production of d_1-cyclopropyl product.

An intermolecular primary deuterium isotope effect of 1.07 ± 0.04 and a minimum intramolecular primary deuterium isotope effect of 3.2 ± 0.5 was measured for the proton abstraction step. Thus, carbon-hydrogen bond cleavage is not a rate-limiting step in the biological cyclopropanation reaction.

A mechanism involving methylation of an olefin followed by 1,3-proton elimination is favoured for the biological formation of cyclopropane fatty acids.

ACKNOWLEDGEMENTS

Many people have helped me in the planning, execution and completion of the work described in this thesis. I wish to thank:

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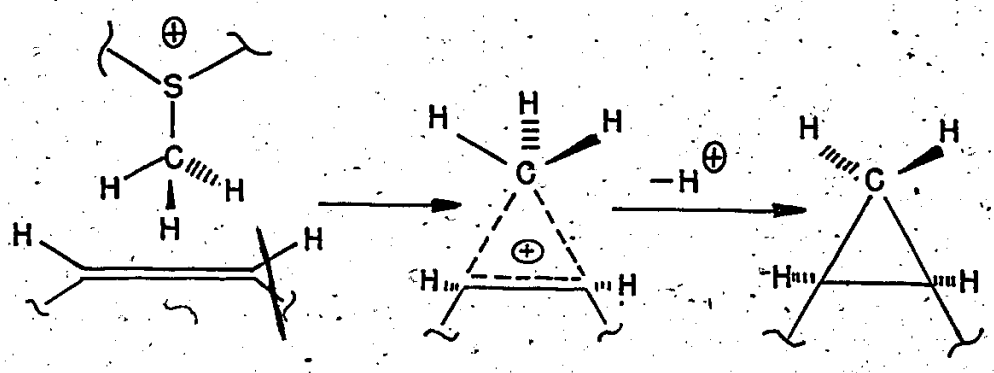
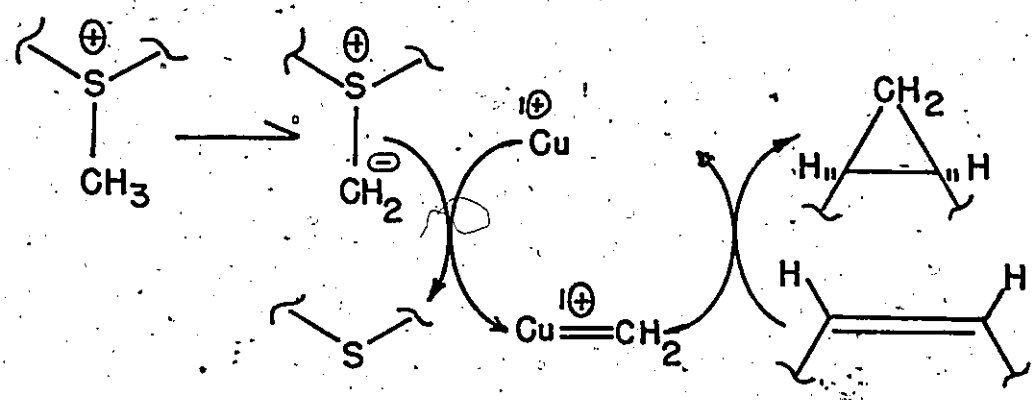
GENERAL INTRODUCTION

The biosynthesis of cyclopropane fatty acids involves the construction of a methylene bridge across the cis double bond of an unsaturated precursor (1). The methylene group is derived from S-adenosyl-L-methionine (S.A.M.) which is known to donate an intact methyl group to a variety of biological nucleophiles, such as thiols, phenols, amines and enols (2). It is commonly held (3) that in the biological cyclopropanation reaction, S.A.M. is able to methylate the weakly nucleophilic double bond, yielding an intermediate carbonium ion, which may lose a proton to form the cyclopropane ring (Scheme 1).



Scheme 1

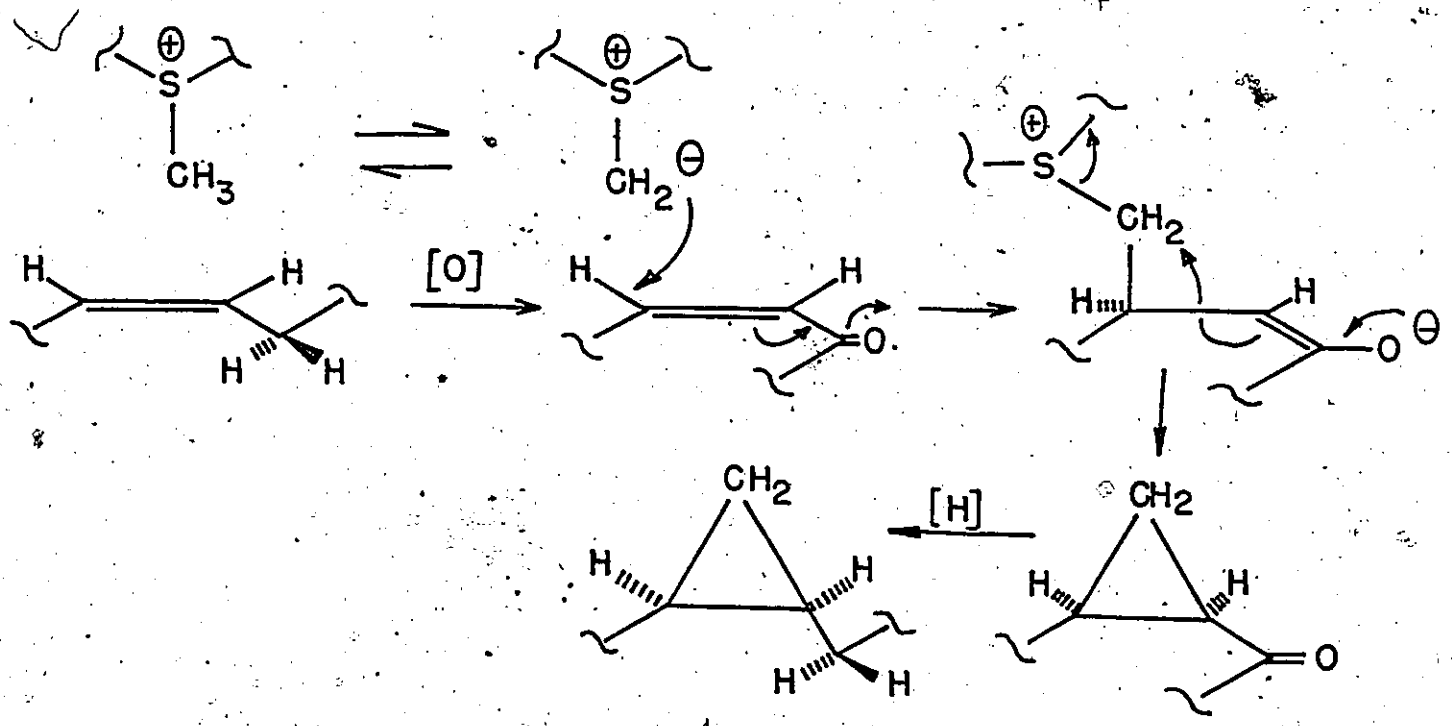
While this mechanism is attractive from many points of view, there are other possibilities. For example, T. Cohen has postulated sulfur ylid formation, followed by transfer of the methylene unit to a metal such as copper, which may then cyclopropanate the double bond in a carbenoid reaction (Scheme 2) (4).



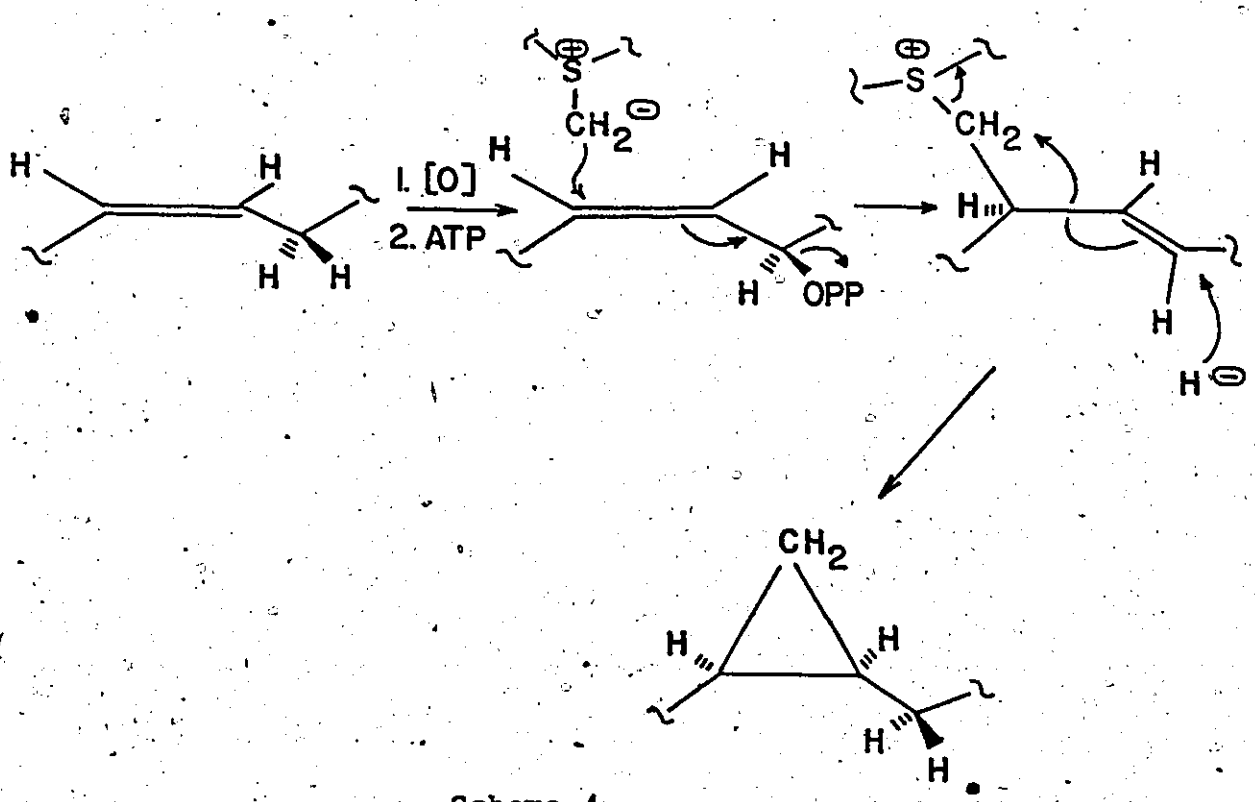
Scheme 2

Another possibility, which has not been previously suggested, is allylic oxidation of the olefin to give an α,β unsaturated ketone, followed by Michael addition of a preformed sulfur ylid. The intermediate so obtained could collapse in the usual manner to yield a cyclopropyl ketone which would have to be reduced to obtain the desired product (Scheme 3).

A closely related variant on the above scheme involves oxidation to an allylic alcohol, which after suitable derivatization might be attacked by a sulfur ylid. Reduction with hydride ion would give the cyclopropane directly (Scheme 4).

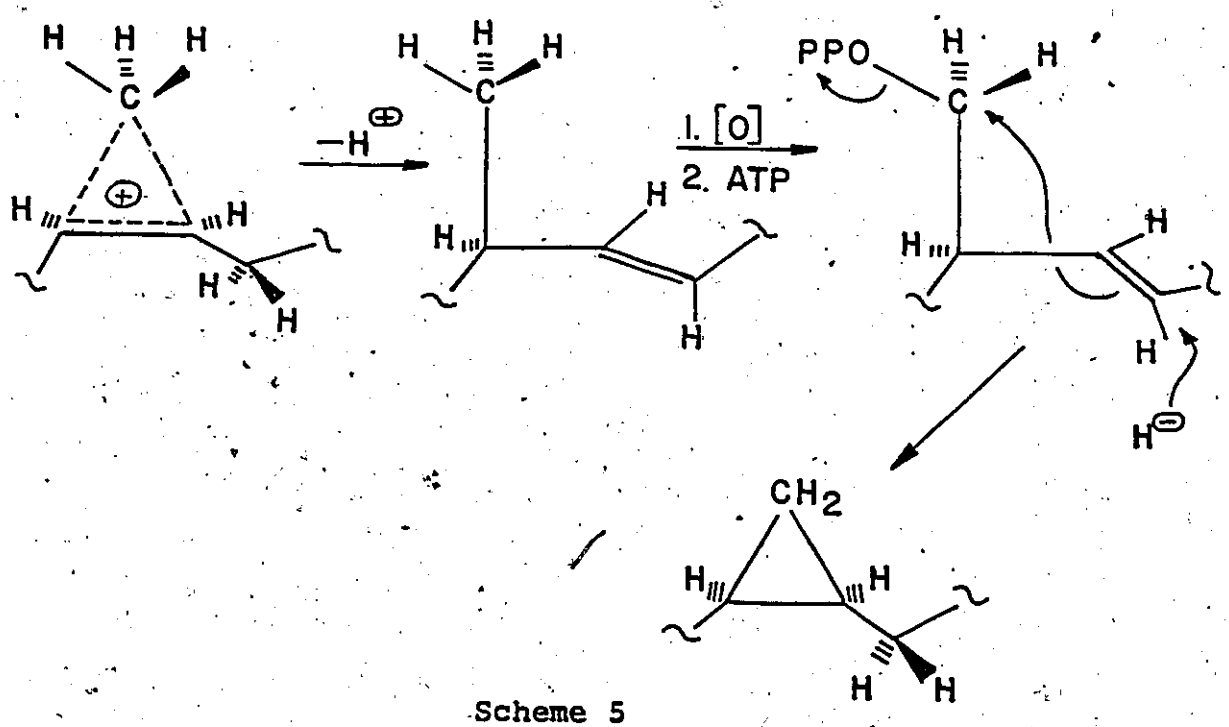


Scheme 3



Scheme 4

Yet another possibility involves quenching of the intermediate carbonium ion generated by methylation of the double bond (see Scheme 1) to form an alkene, which after methyl group oxidation and derivatization, might be attacked by hydride ion at the double bond to yield the cyclopropane (Scheme 5).



Scheme 5

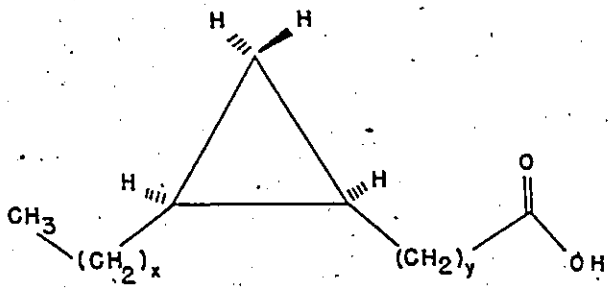
It was the purpose of this investigation to distinguish among the above mechanistic possibilities. In particular, the techniques of deuterium labelling combined with mass spectrometry and nuclear magnetic resonance were to be used in order to check for loss of allylic hydrogen from the olefinic substrate during cyclopropanation. Furthermore, it was considered appropriate to repeat earlier labelling experi-

ments which had shown that two deuterons from methionine-methyl-d_3 were incorporated into the cyclopropane fatty acid (5), and that the unsaturated precursor, deuteriated at the vinyl positions, did not suffer loss of label during cyclopropanation (6). In neither experiment was the position of the label in the biosynthetic product unambiguously determined. Also, it was not reported whether partial exchange had occurred in the methionine-methyl-d_3 feeding; this information was deemed important in deciding at what stage the deuteron (proton) is lost in the biological cyclopropanation reaction: namely, before or after carbon-carbon bond formation.

HISTORICAL INTRODUCTION

A Cyclopropane fatty acids: Structure, distribution and function.

Cyclopropane fatty acids occur in many bacteria as esters of cell membrane phospholipids. They have also been found in some seed oils, along with the corresponding cyclopropene fatty acids and in some protozoa (7). Typical structures are shown in Figure 1.



x = 5, y = 9: lactobacillic acid

x = 7, y = 7: dihydrosterculic acid

x = 5, y = 7: 9,10-methylenehexadecanoic acid

Figure 1. Some typical cyclopropane fatty acids.

Only in the case of lactobacillic acid, isolated from Brucella melitensis, has the absolute configuration of the cyclopropane ring been shown to be 11R,12S (see Figure 1) (8). Circumstantial evidence points to dihydrosterculic acid having the same configuration (9).

The precise biochemical role of the cyclopropyl fatty

acids has not been clearly defined. Some uncertainty exists in the literature regarding the effect of cyclopropanation on the physical properties of the cis-olefinic fatty acid containing phospholipids. Recently, workers in this area have suggested that there is, in fact, very little effect* (10,11). An antioxidant role seems out of the question since anaerobes and aerobes both possess cyclopropane acids. That cyclopropane formation is a means of disposing of excess S.A.M. is not in accord with the observation that S.A.M. levels do not influence the rate of cyclopropane biosynthesis (12). All things considered, especially the fact that cyclopropane phospholipids are not further metabolized, it seems likely that the cyclopropyl group plays some subtle structural role in the cell membrane.

B Biosynthesis of cyclopropane fatty acids

Very soon after K. Hofmann isolated the first cyclopropane fatty acid (lactobacillic acid) in 1950 (13), the first biogenetic hypothesis for its formation *in vivo* was put forward by E. Kosower (14). He reasoned that suitable

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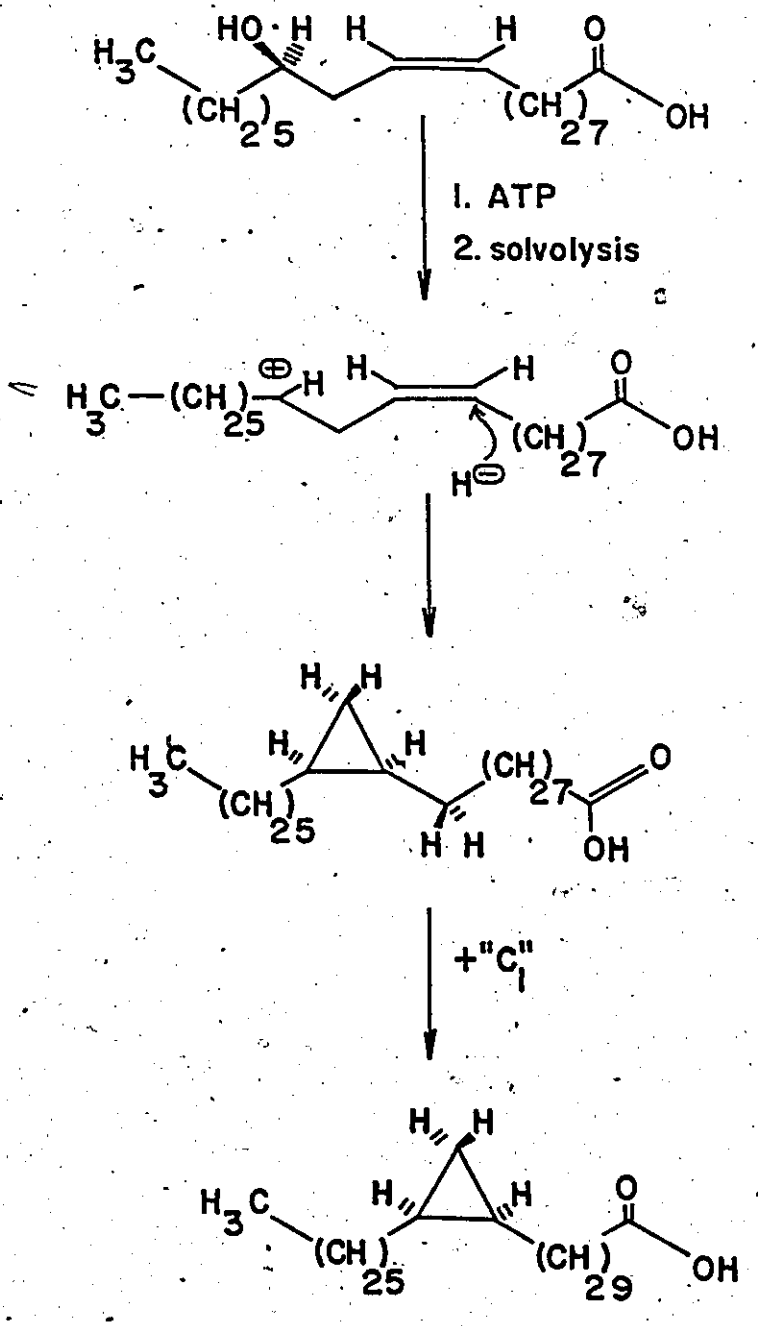
* Silvius and McElhaney have found the presence of the cyclopropane group in the fatty acid acyl sidechains to cause only a slight increase in ordering of the phospholipid bilayer (11). Cronan *et al.* failed to find any difference in the thermotropic properties of cyclopropane and cis-double bond containing phospholipids (10).

derivatization of ricinoleic acid, followed by solvolysis, reduction and chain elongation could lead to the C-19 cyclopropane acid (Scheme 6).



Scheme 6

It is interesting to note that although this hypothesis turned out to be incorrect, it did predict the correct position of the cyclopropane ring in lactobacillic acid (the complete structure was determined in 1960) (15).

In 1957, Hofmann noted that the addition of cis-vaccenic acid (cis-11-octadecenoic acid) to the bacterial growth medium stimulated production of lactobacillic acid. Furthermore, when the latter was supplied in the medium, no vaccenic acid was biosynthesized (16). The precursor role of cis-vaccenic acid was confirmed by W. O'Leary who fed cis-vaccenic-1-^{14}C and oleic-1-^{14}C acid and obtained a ^{14}C labelled cyclopropane acid of similar specific activity (17). Finally, it was unambiguously shown by Hofmann that methionine-methyl-^{14}C, and to a much lesser extent, formate-^{14}C, was incorporated specifically into the methylene bridge of lactobacillic acid (18). Thus, it seemed likely that a C_1 unit was added across the double bond of vaccenic acid to form, in some unprecedented fashion, the cyclopropyl ring of lactobacillic acid.

C Enzymology of cyclopropane synthetase

In order to study the cyclopropanation reaction more closely, J.H. Law and coworkers attempted to isolate the enzyme responsible for this unique transformation. Although they did not succeed, work with purified extracts revealed some useful information. Earlier on, working with mutants of Aerobacter aerogenes, O'Leary had shown that S-adenosyl-L-

methionine was a more efficient "methylene donor" than methionine (19). Zalkin et al. confirmed this result by demonstrating that S.A.M. was necessary for cyclopropane formation to occur in vitro (20). N-Methyl-tetrahydrofolate was inactive as a "methylene donor" (21). The next point to be determined was the nature of the olefinic substrate. Free fatty acids were not cyclopropanated. Phospholipids containing olefinic fatty acids, such as dioleyl phosphatidyl ethanolamine, when dispersed to form micellar solutions did act as substrates. The enzyme would accept a variety of phospholipids except for phosphatidyl choline derivatives. Oleyl sidechains linked to positions 1 and 2 of glycerol-3-phosphate by non-hydrolyzable ether bonds were cyclopropanated showing that hydrolysis of phospholipids to free fatty acids followed by cyclopropanation and recondensation had not occurred in the previous experiments (22). Thus, the cyclopropane synthetase catalyzed reaction involves the interaction of phospholipid micelles with a protein; in vivo, the reaction probably takes place at the cell membrane (23). Recent work by Taylor and Cronan showed that the enzyme can be stabilized by phospholipid vesicles (24).

The enzyme which Law studied was isolated from Clostridium butyricum and was unique in that although it did cyclopropanate the beta fatty acid chain, it preferred to react with the fatty acid at the gamma position (25). Most cyclopropane-containing phospholipids which have been isolated

from other sources have the cyclopropyl sidechain at the beta position. This may simply be due to the fact that the precursor olefin is usually found at that position. The naturally occurring L-glycerol phosphatides are an absolute requirement for the cyclopropane synthetase (22). Evidence from *in vivo* experiments points to the fact that only the 9,10- and the 11,12-cis-octadecenoic fatty acids are cyclopropanated. Hexadecenoic isomers have not been studied as thoroughly, although it has been shown that the 9,10-isomer is strongly preferred (26). Ohlrogge *et al.* have suggested that two separate enzymes may exist; one which recognizes the carboxyl end of the fatty acid and the other the methyl end (27).

The other product of the cyclopropanation reaction, S-adenosyl-L-homocysteine (S.A.H.) acts as a strong inhibitor of the enzymic reaction; this seems to be a general feature of S.A.M. transmethylations. In the crude extracts with which Law worked, S.A.H. was degraded by S.A.H. nucleosidase to adenine and ribosylhomocysteine (28). A summary of the information gained so far is presented in Figure 2.

D Mechanisms of biological cyclopropanation

In 1963, J.H. Law and coworkers fed methionine-methyl-d_3 to *E. coli* and isolated a cyclopropane product which was shown by mass spectrometry to be dideuterated (5). On the basis of this result, Law argued that biological cyclopropanation might follow a course similar to E. Corey's newly discovered

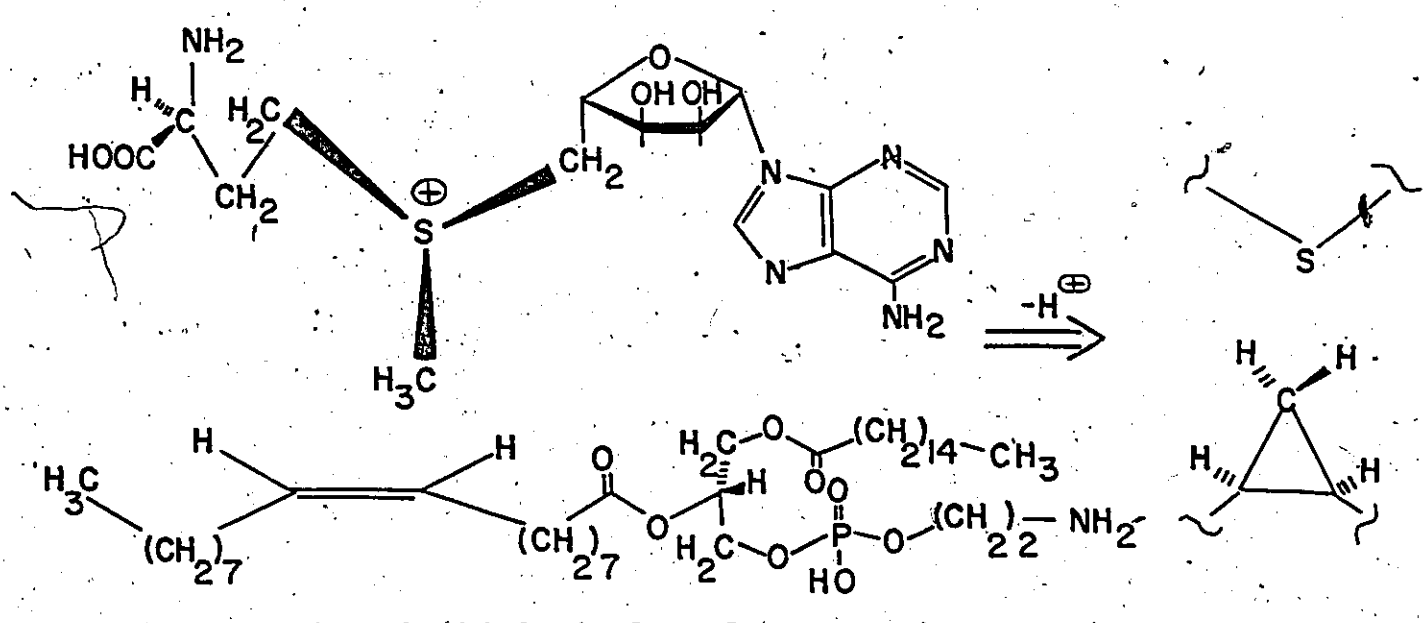
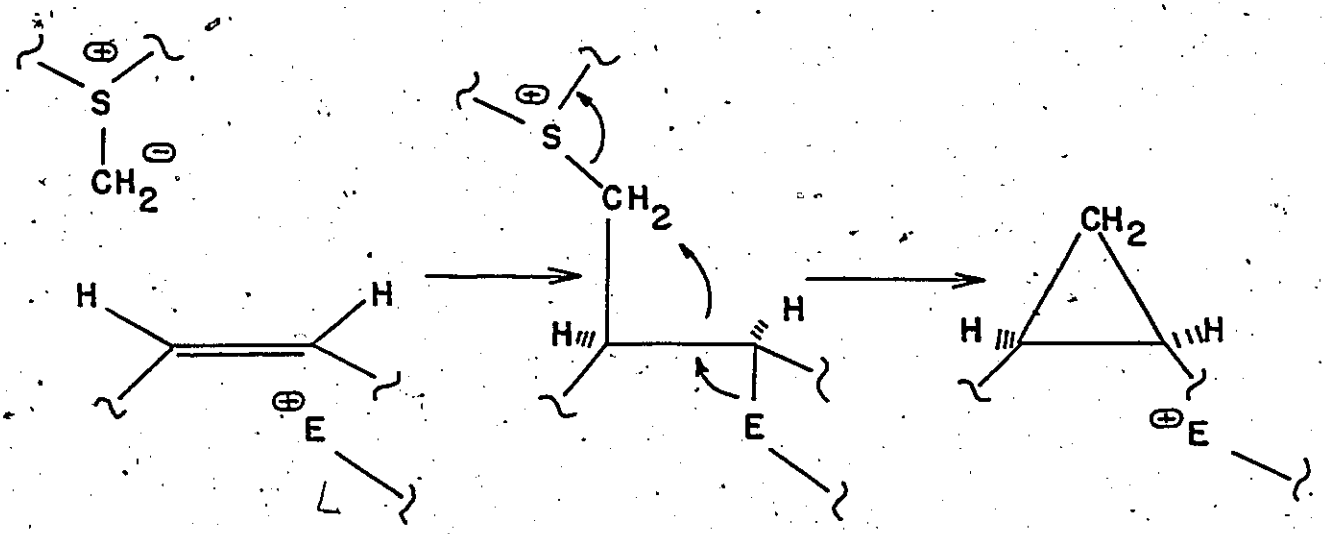


Figure 2. The biological cyclopropanation reaction.

synthetic reaction (29), involving the addition of a sulfur ylid to the double bond of an α,β-unsaturated ketone. The analogy was a poor one since sulfur ylids do not add to an unactivated double bond such as is found in cis-vaccenic acid. E. Lederer, in his 1964 review on C-alkylation (30), suggested that the sulfonium ylid formed from S.A.M. might either alpha eliminate to yield a free carbene, capable of cyclopropanating an isolated double bond, or, might attack a double bond polarized by an appropriately situated enzyme group (Scheme 7).

The nature of the enzyme group capable of accepting electrons and redonating them was not defined. At about the



Scheme

same time, O'Leary raised the possibility of there being discrete intermediates in the cyclopropanation reaction, although he did not give chemically reasonable mechanisms for their formation (31) (Figure 3).

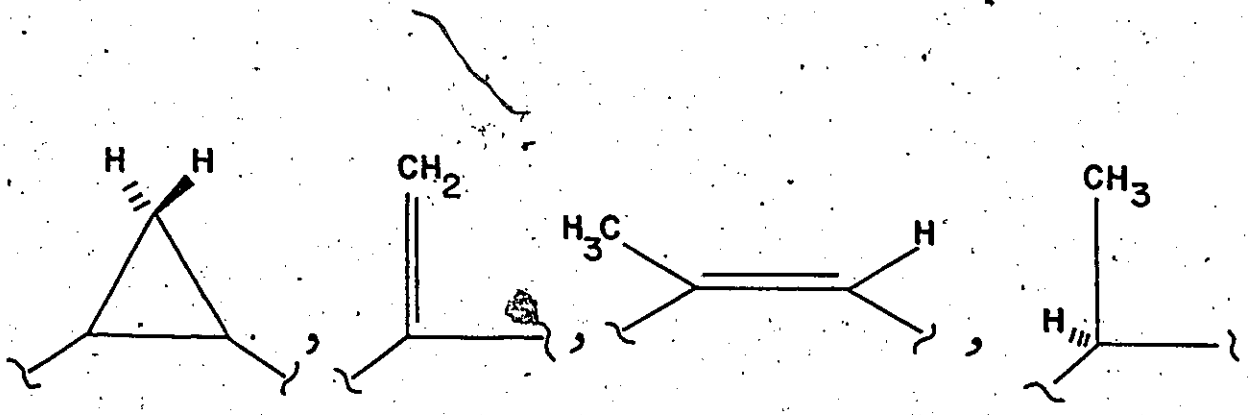
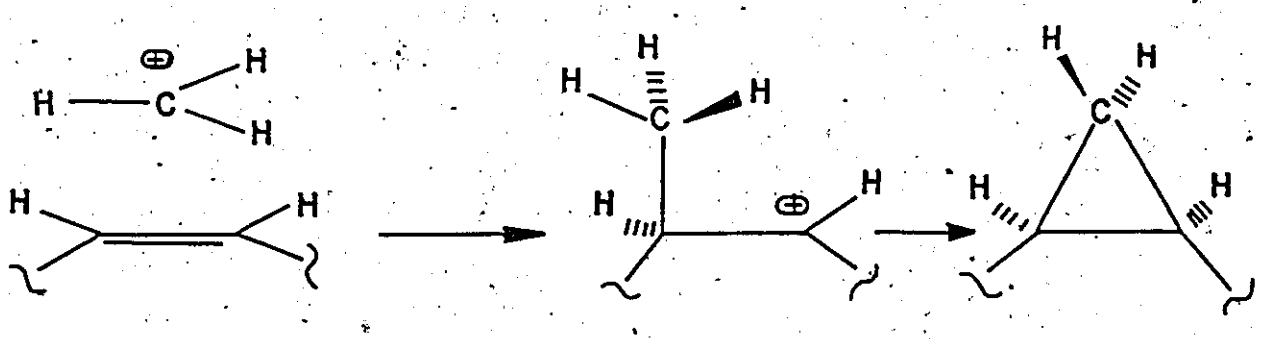


Figure 3. Some possible intermediates in the biological cyclopropanation reaction.

Law ruled out some of these suggestions by feeding oleic acid-9,10-d_2 to L.plantarum and isolating a biosynthetic cyclopropyl product which he found had retained the two deuterons (6). He also showed by a mass spectrometric method that the 9,10-olefin was converted to the 9,10-cyclopropane and not to the 11,12-isomer as had been previously suggested (17).

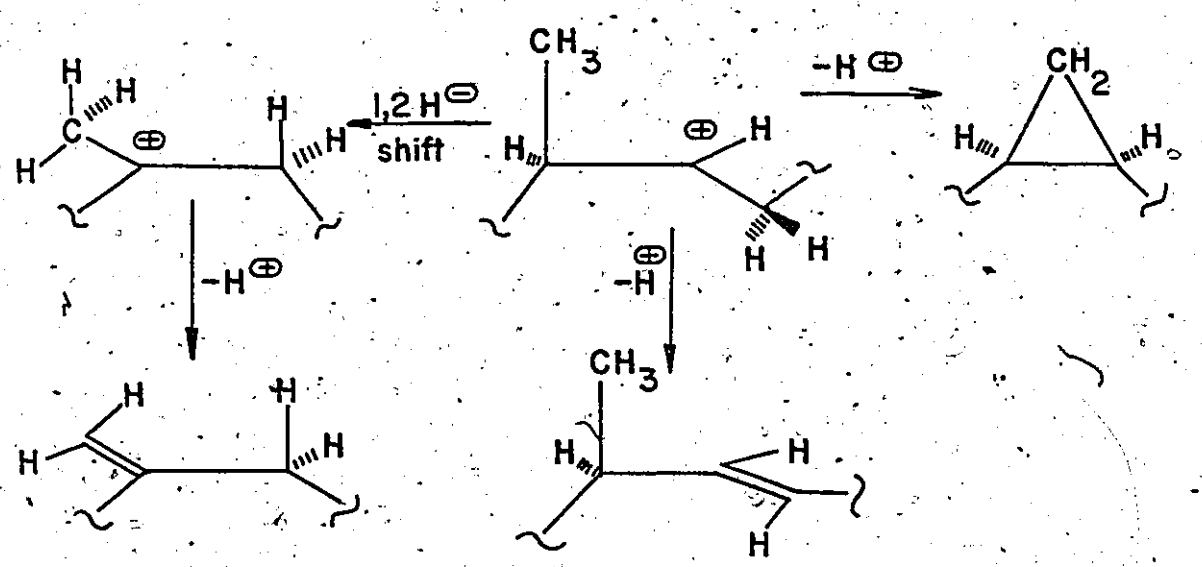
In 1964, R.C. Greene suggested that a polarized double bond might attack a methyl carbonium ion to yield another carbonium ion, which should "theoretically be able to displace a proton from the entering methyl group" (32) (Scheme 8).



Scheme 8

Actually, the idea of an isolated double bond attacking a "methyl cation" was apparently first suggested in 1962 by A.J. Birch (33), who correctly predicted the origin of ethyl sidechains in phytosterols as arising from a double methylation process. In 1969, Lederer provided a unified theory of

C-alkylation reactions in which the cyclopropane ring was thought to arise from the same intermediate carbonium ion which also leads to exomethylene and allylic methyl group formation (34) (Scheme 9).



Scheme 9

The sulfur ylid proposal was revived in 1974 by T. Cohen who considered a metal carbenoid mechanism to be a likely possibility (4) (see Scheme 2). He carried out model studies to show that methyl sulfonium salts could be easily deprotonated under mild conditions and that a sulfur ylid could cyclopropanate alkenes in the presence of copper salts.

Since then, no further suggestions about the mechanism of biological cyclopropanation have been made in the literature.

Although the regulation and biochemical role of cyclopropane fatty acid biosynthesis has been studied, no detailed mechanistic work has been reported for the last 13 years.

RESULTS AND DISCUSSION

A. INTRODUCTION

The study of the biological cyclopropanation reaction using deuterium labelling techniques requires a biological system, which will incorporate labelled precursors efficiently. The use of the micro-organism, Lactobacillus plantarum, ATCC 8014, was found to be convenient in this regard. The growth medium for this bacterium could be altered so that incorporation of methyl-labelled methionine, or labelled olefinic fatty acids, into the cyclopropane fatty acids, was essentially 100%. Since the organism will cyclopropanate both cis-11,12 and cis-9,10-octadecenoic acid (6), it was decided to prepare and feed the much more readily available, labelled 9,10-isomer.

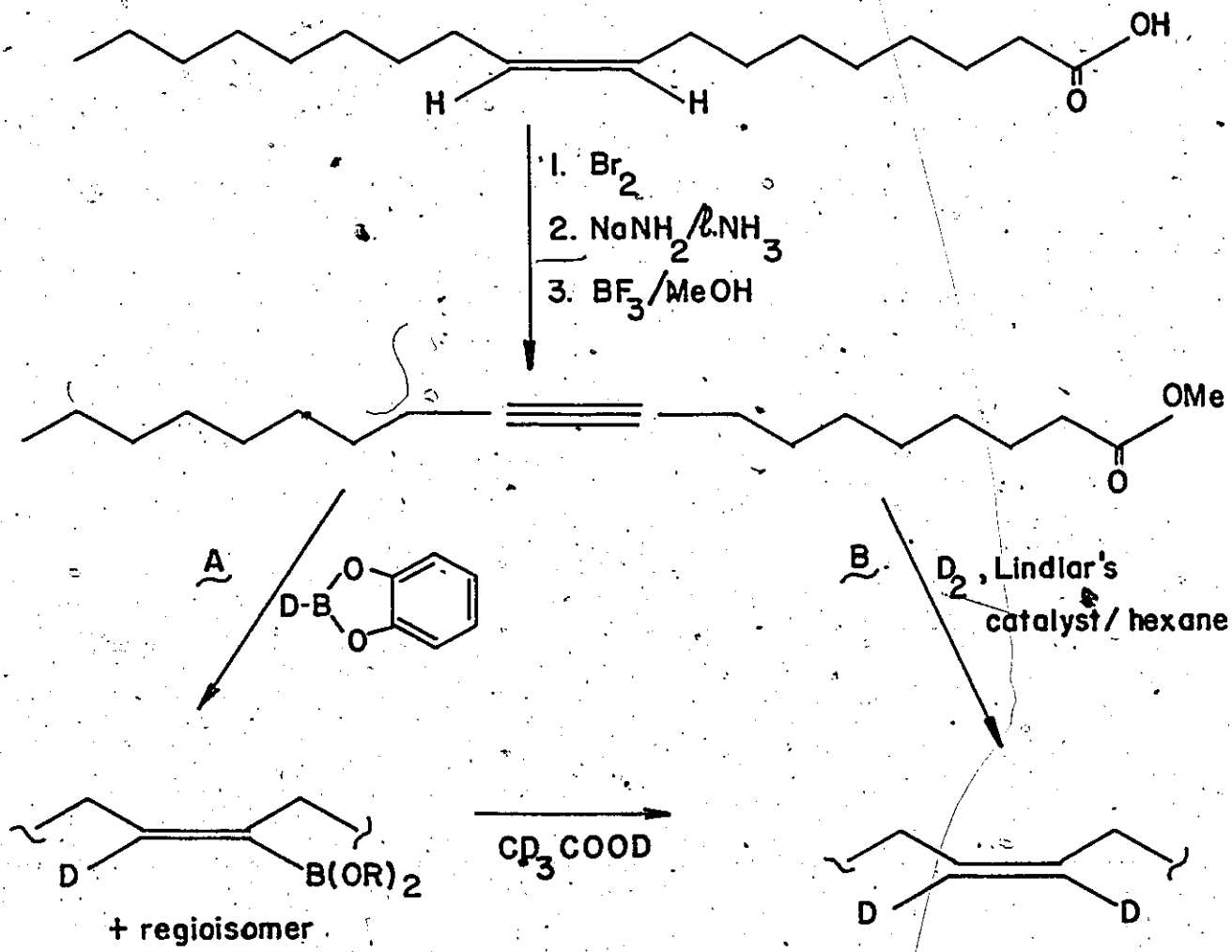
Methionine-methyl-d_3 was fed to the micro-organism to determine if removal of a deuteron to form the methylene unit was reversible or not; if it was, some d_1 and d_0-cyclopropane product might accompany the expected d_2-species. Methionine-methyl-d_2 and methionine-methyl-d_1 were prepared and used to determine the intramolecular deuterium isotope effect for the proton-abstraction step. An intermolecular deuterium isotope effect was measured by feeding a 1:1 mixture of trideuterio and non-deuteriated methionine. By assembling the evidence obtained from the above experiments, it was hoped that a coherent

mechanistic picture of the biological cyclopropanation reaction might be obtained.

B. SYNTHESIS OF DEUTERIATED FATTY ACIDS

(i) Methyl cis-9-octadecenoate-9,10-d_2

The two routes that were investigated for the preparation of the title compound from readily available methyl 9-octadecynoate (35,36) are outlined in Scheme 10.



Scheme 10

Route A was investigated first, because it was anticipated that pure cis-olefin would be obtained, without scrambling of the label (37). This indeed turned out to be the case, although the resulting deuterium content in the olefin was low (d_0, 3%; d_1, 30%; d_2, 67%). Use of non-labelled catecholborane and deuteriated acetic acid gave rise to a mono-deuteriated olefin which was 90% d_1. Thus, the catecholborane-B-d, prepared from BD_3/THF, was probably only partially (75%) deuterated at boron.

The desired compound was obtained, with high deuterium content (d_0, 1%; d_1, 3%; d_2, 96%) and accompanied by only traces (2%) of the trans compound, when pathway B was followed. The use of ethyl acetate (38) or ethanol-O-d as solvent led to an olefin with lower d_2 content (90-93%) and containing more trans material (6-8%). It has been suggested that trace amounts of acidic impurities catalyse isomerization of the double bond (39). The position of the double bond was not checked; other workers have found no shift of the double bond when using Lindlar's catalyst (40). The title compound was obtained in 23% overall yield from oleic acid. The above method represents a substantial improvement over catalytic reduction using quinoline as a catalyst poison, where ca. 15% d_1-olefin is produced (41).

Various NMR techniques were used to confirm the position of the deuterium label. Comparison of the ^1H spectrum of methyl

oleate and methyl oleate-9,10-d_2 (Figures 4 and 5, respectively) showed that in the labelled material, only traces of vinyl-proton absorption remained at δ 5.3; furthermore, the allylic resonance at δ 2.0 had been converted into an unresolved triplet as expected. In the ^{13}C NMR spectrum, the signal of the vinyl carbon atoms at 130 ppm, present in the unlabelled analogue, had disappeared (Figures 6 and 7). An upfield β isotope shift (0.1 ppm, (42)) was observed at the allylic carbon atoms (see Table 1, p. 32). In the ^{2}H NMR spectrum (Figure 8) only traces of deuterium at carbon atoms other than the vinylic (δ 5.3) were found.

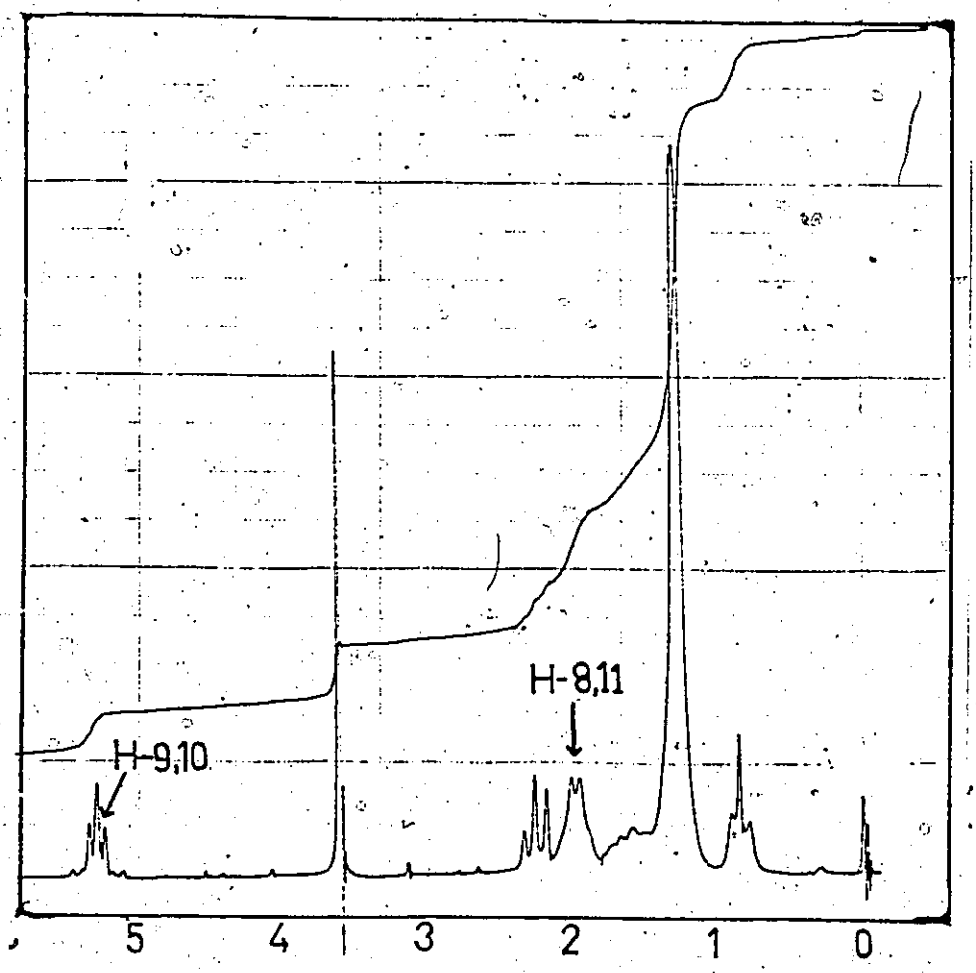


Figure 4. ^{1}H NMR spectrum of methyl oleate.

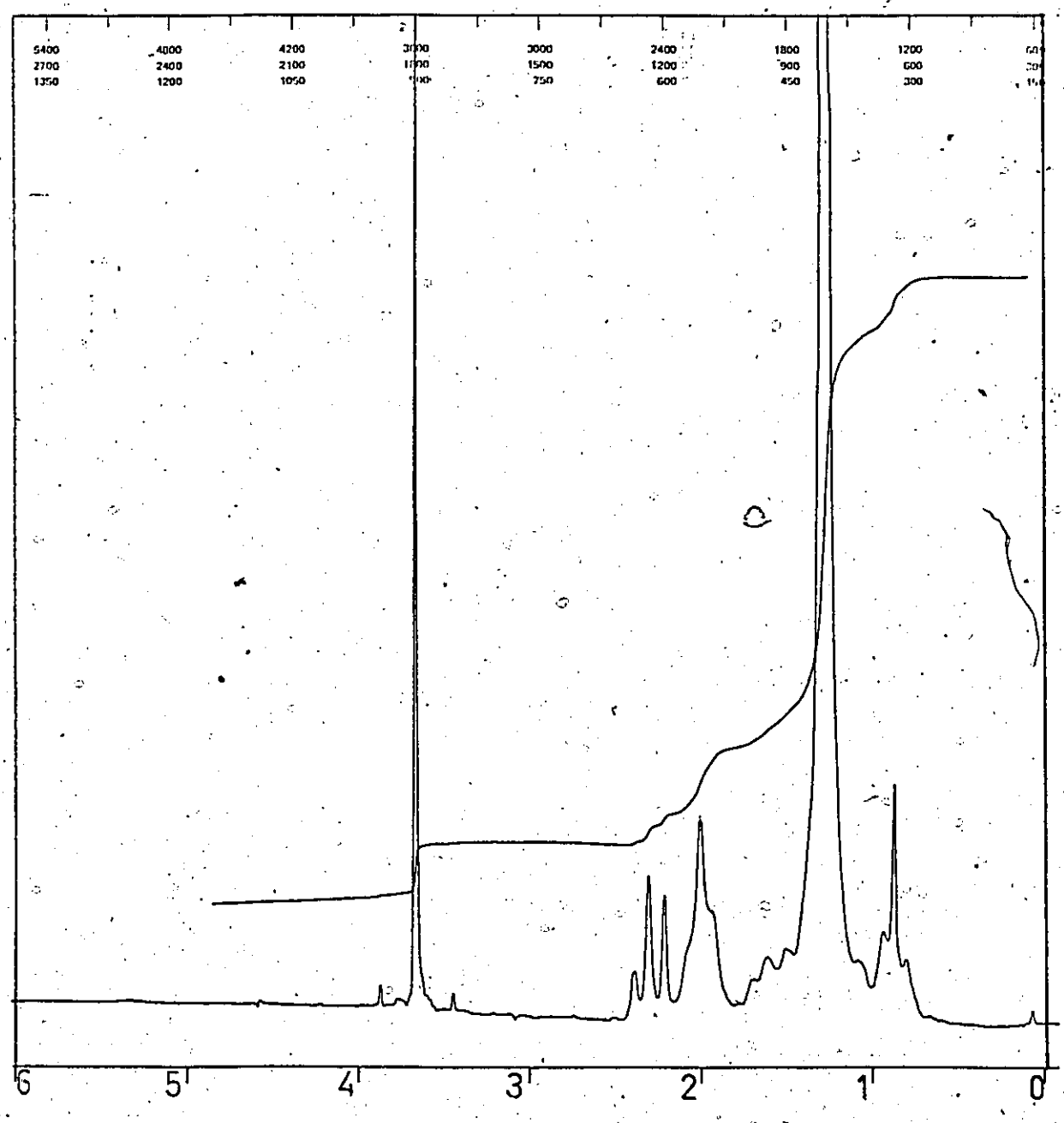


Figure 5. ^{1}H NMR spectrum of methyl oleate-9,10-d_2.

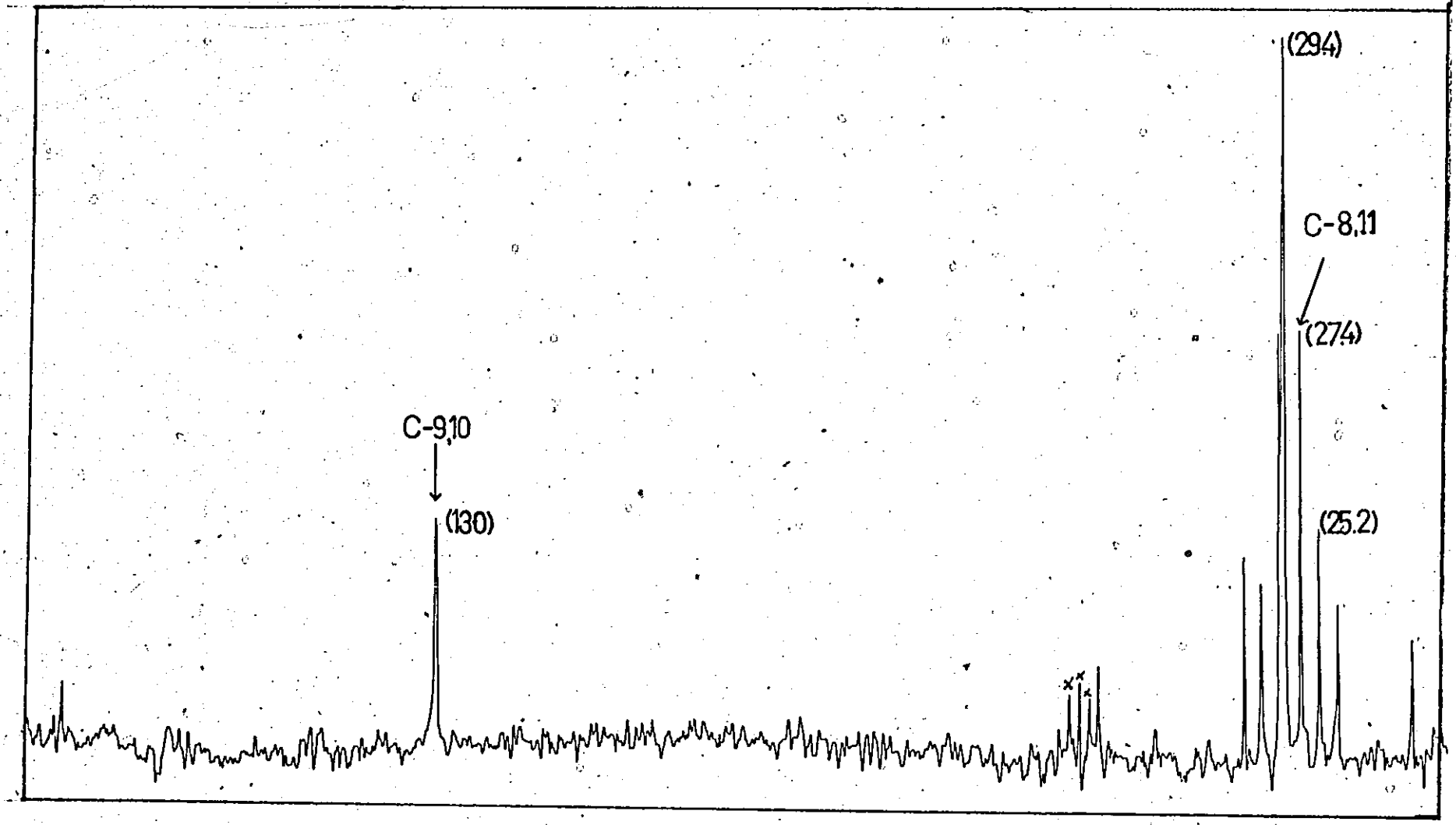


Figure 6. ^{13}C NMR spectrum of methyl oleate.
(solvent peaks are marked with an x).

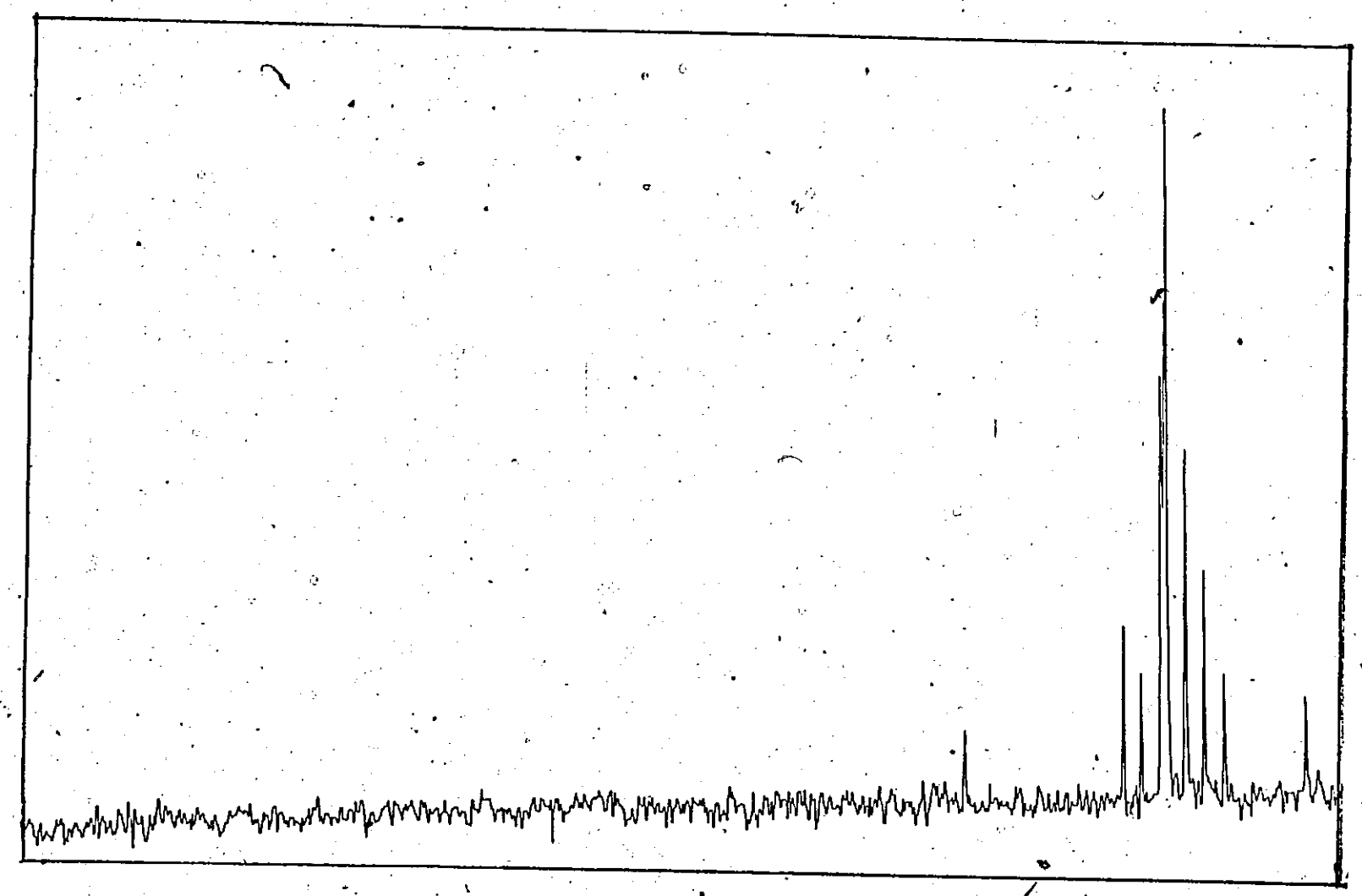


Figure 7. ^{13}C NMR spectrum of methyl oleate-9,10-d_2.

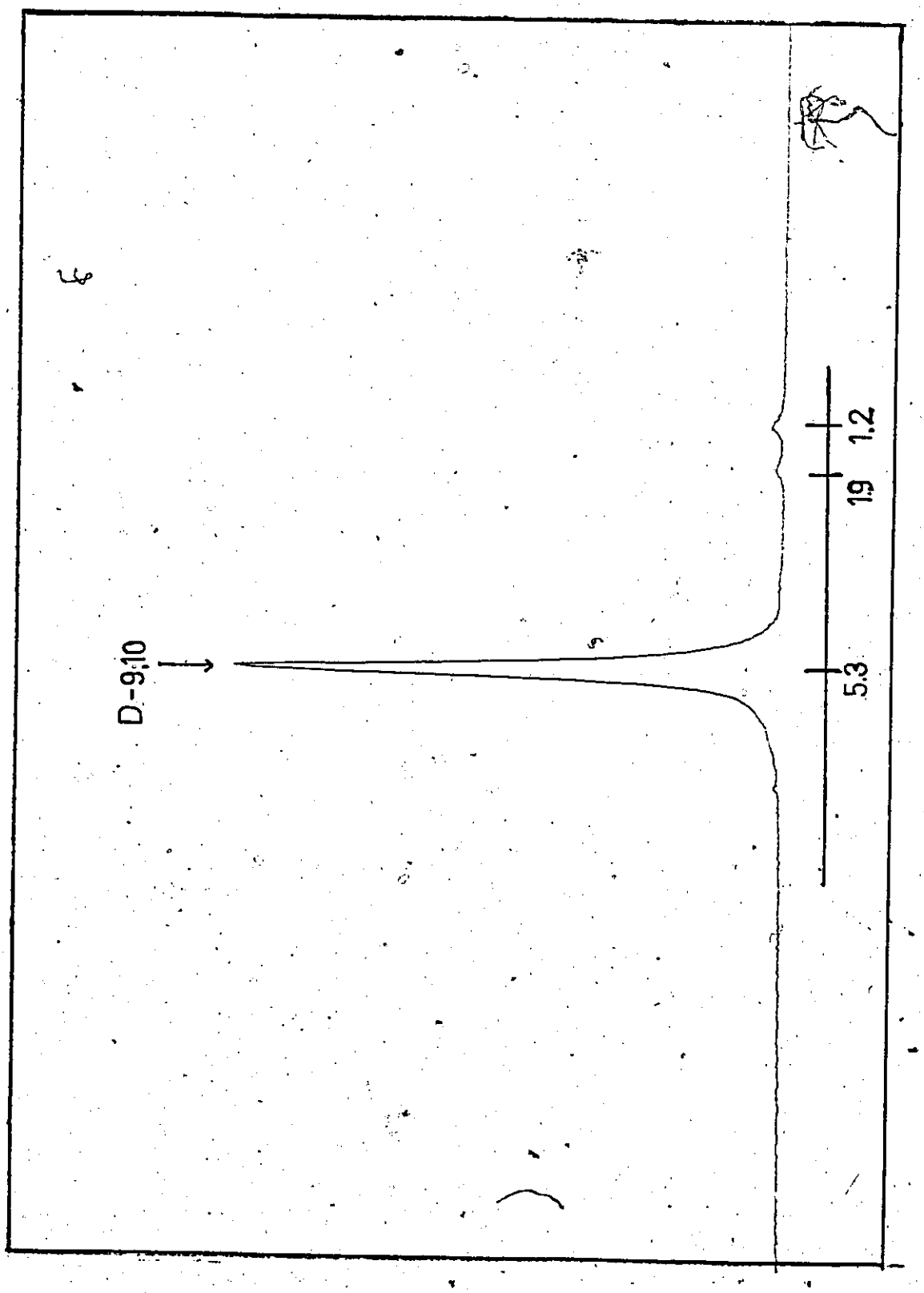
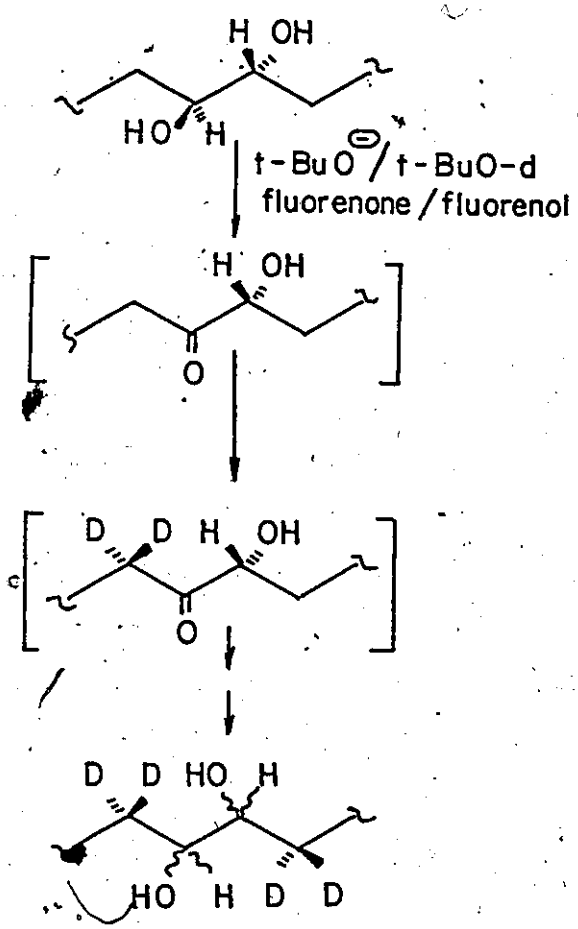


Figure 8. ^2H NMR spectrum of methyl oleate-9,10-d_2.

(iii) Methyl cis-9-octadecenoate-8,8,11,11-d_4

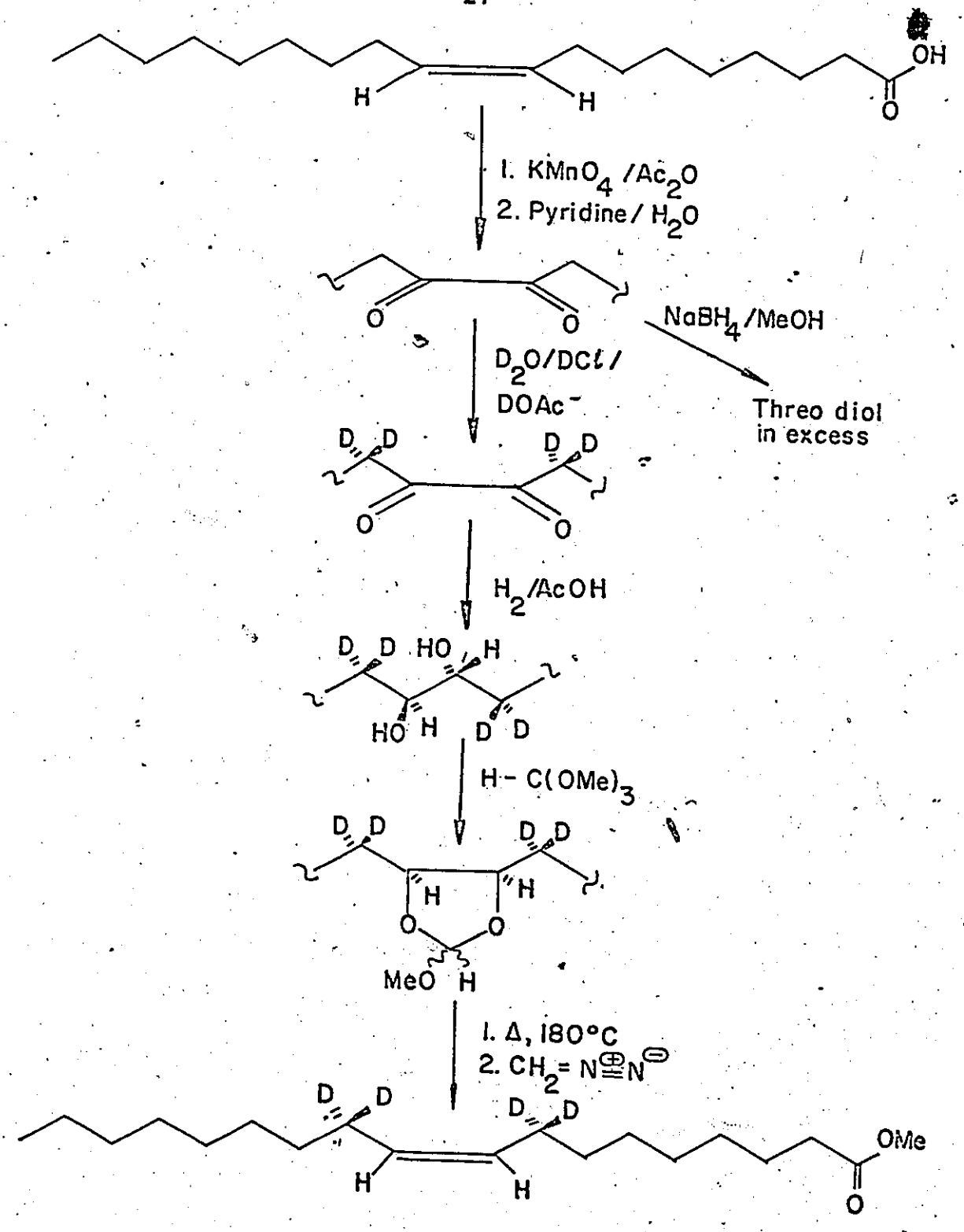
At the outset of this work, there was only one method in the literature for the preparation of the title compound. It involved eight steps and gave a product containing 30% of the trans isomer (43). Accordingly, a more satisfactory method was sought. The first attempt in this direction is outlined in Scheme 11.



Scheme 11

The 9,10-diol, obtained by basic permanganate oxidation of oleic acid (44), was subjected to racemization conditions (45) in the expectation that the intermediate ketol would exchange. Unfortunately, exchange at the 8,11 positions was not observed. However, from the 9,10-diketone, a successful synthetic route to the title compound was developed (Scheme 12). The 9,10-diketone, obtained by permanganate-acetic anhydride oxidation of oleic acid (46), underwent facile exchange at the 8,11 positions, at 65°C, in a mixed solvent system, generated by adding D_2O to acetyl chloride and acetic anhydride. Catalytic reduction of the exchanged diketone yielded mainly the erythro diol, which was freed from the threo isomer by two recrystallizations from ethyl acetate. The predominant formation of erythro diol might be explained by postulating a cis-enediol intermediate that undergoes cis-hydrogenation of the double bond. In contrast, reduction of the diketone using sodium borohydride, gave an excess of the threo diol; fractionation of the mixture by continuous extraction with ether, had been reported to separate the two diastereoisomeric diols (47). However, this method could not be reproduced.

The deoxygenation of the erythro diol was successfully carried out by using methyl orthoformate to generate the 1,3-dioxalan, which was heated to eliminate CO_2 and methanol; partially unmethylated acid served as a catalyst for the decomposition (48). The title compound was thus obtained in 20%


OH
O
H
H
1. KMnO4 /Ac2O
2. Pyridine/ H2O
O
O
NaBH4 /MeOH
Threo diol
in excess
D2O/DCl/
DOAc
D D
D D
O
O
H2/AcOH
D D HO H
HO H D D
H- C(OMe)3
D D
D D
H
H
O
O
MeO H
1. Δ, 180°C
2. CH2= N⊕N⊖
D D
D D
H
H
OMe
O

Scheme 12

overall yield from oleic acid. The stereochemistry of the double bond was determined to be 100% cis within experimental error and the distribution of deuterium was measured to be as follows: d_4, 79.1%; d_3, 15.1%; d_2, 4.0%; d_1, 1.4%; d_0, 0.4%.

That all the deuterium was at the allylic positions was shown by the following evidence. In the ^1H NMR spectrum (Figure 9), the signal at δ 2.0 present in the unlabelled compound, was absent. The signal of the vinyl hydrogens had sharpened into a singlet as expected. The signal assigned to the allylic carbon atom at 27.4 ppm in the unlabelled compound (Fig. 6) had disappeared in the ^{13}C NMR spectrum of the labelled material (Figure 10) and a β isotope shift (0.2 ppm) was observed for the signals assigned to the carbon atoms at C-7 and C-12 (see Table 1, p. 32). Only one peak at δ 1.8 was present in the ^2H NMR spectrum (Figure 11) as expected.

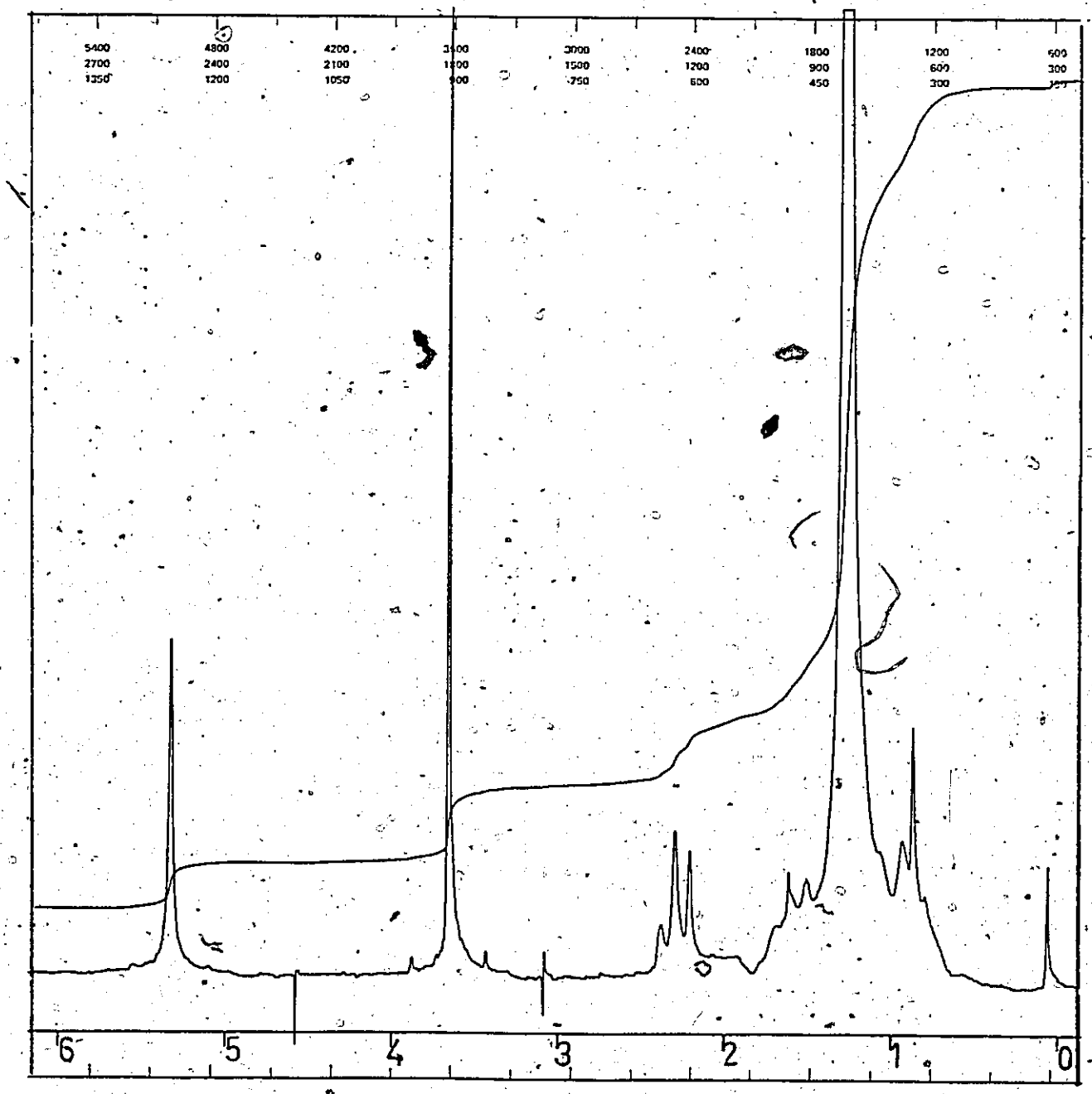


Figure 9. ^{1}H NMR spectrum of methyl oleate-8,8,11,11-d_4.

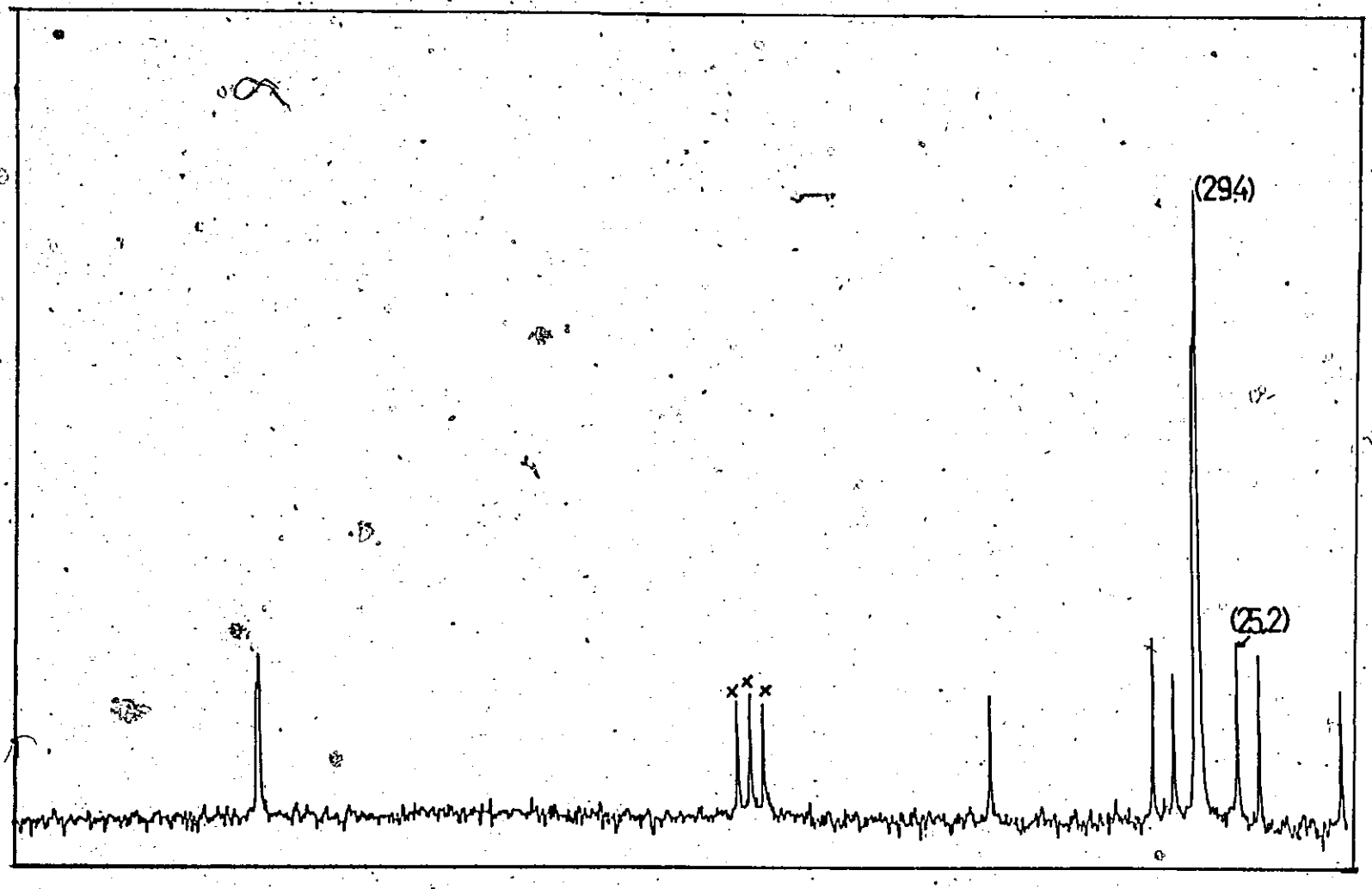


Figure 10. ^{13}C NMR spectrum of methyl oleate-8,8,11,11-d_4.

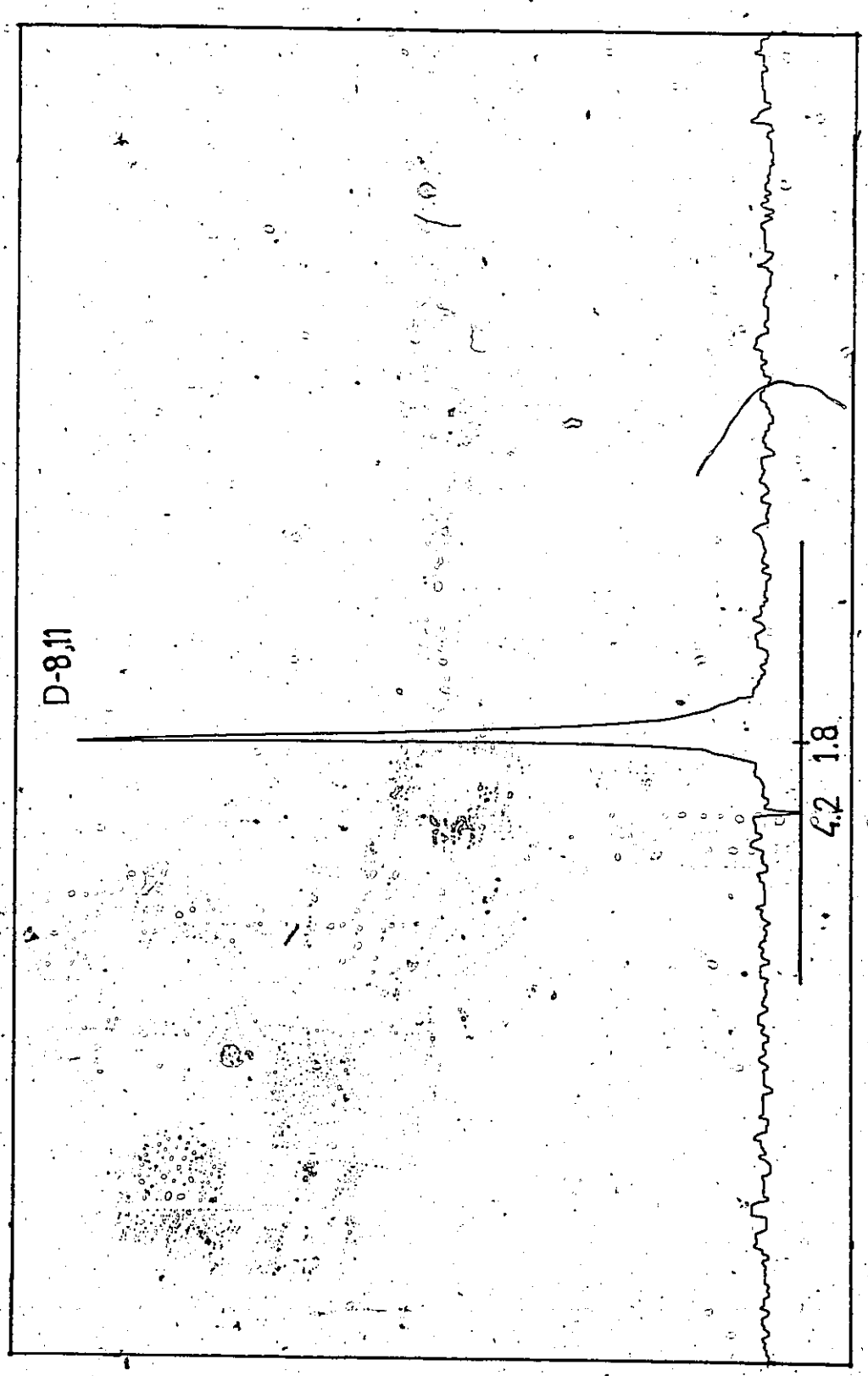


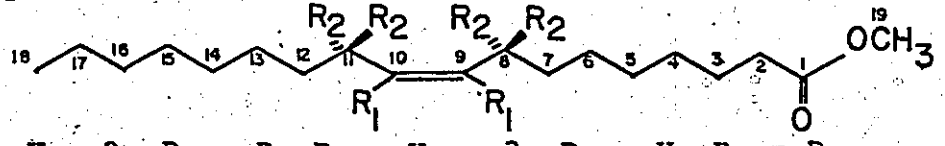
Figure 11. ^2H NMR spectrum of methyl oleate-8,8,11,11-d_4.

Table 1.

^{13}C chemical shifts of deuteriated and non-deuteriated Methyl cis-9-octadecenoates

Compound / Carbon #	1	2	3
1	174.3	(-)	(-)
2	34.2	34.2	34.3
3	25.2	25.2	25.2
4	29.4	29.4	29.4
5	29.4	29.4	29.4
6	29.4	29.4	29.4
7	30.0	30.0	29.8
8	27.4	27.3	ABSENT
9	130.1	ABSENT	130.1
10	130.2	ABSENT	130.2
11	27.4	27.3	ABSENT
12	30.0	30.0	29.8
13	29.4	29.4	29.4
14	29.4	29.4	29.4
15	29.4	29.4	29.4
16	32.2	32.2	32.2
17	22.9	22.9	22.9
18	14.2	14.2	14.3
19	51.4	51.3	51.6

(-) denotes peak of very low intensity.



1 $R_1 = R_2 = H$, 2 $R_1 = D$, $R_2 = H$, 3 $R_1 = H$, $R_2 = D$

C. FEEDING EXPERIMENTS WITH LABELLED FATTY ACIDS

The two deuteriated fatty acids were fed to the microorganism at a concentration of 0.06 mM on a 10 L scale. Approximately 5 mg of cyclopropane fatty acid were isolated in each experiment (representing a 2.5% conversion). For comparison purposes, the Simmons-Smith reaction (49) was employed to prepare deuteriated cyclopropanes from the corresponding deuteriated olefins. Examination of the synthetic and biosynthetic samples by ms indicated that label was not lost in either feeding experiment (Table 2).

The presence of the small amounts (2-3%) of unlabelled cyclopropane fatty acid in the biosynthetic products was probably caused by residual olefin fatty acid biosynthesis.

The label in the biosynthetic product from both feeding experiments was located by NMR methods. The ^{1}H NMR of methyl dihydrosterculate is shown in Figure 12. The peaks of interest are the multiplet at δ 0.66 (3H) and the multiplet at δ -0.26 (1H). These resonances have been assigned by Longone and Miller (50). The cis protons at C-9 and C-10 and the bridgehead proton trans to the two alkyl substituents resonate together at ca. δ 0.6 while the bridgehead proton cis to the 2 alkyl substituents is observed at ca. δ -0.3. Specific deuterium labelling at the bridgehead would unambiguously confirm these assignments. In the ^{1}H NMR spectrum (Figure 13) of the biosynthetic cyclopropane derived from oleic acid-9,10-d_2, a pair of doublets (J_{gem} = ca. 4 Hz) at δ 0.55 (1H) and

Table 2

Deuterium content of Synthetic and Biosynthetic Cyclopropane Fatty Acids derived from oleic acid-9,10-d_2 and oleic acid-8,8,11,11-d_4

	Synthetic 9,10 d_2-Cyclopropane Acid	Biosynthetic 9,10 d_2-Cyclopropane Acid	Synthetic 8,8,11,11 d_4 Cyclopropane Acid	Biosynthetic 8,8,11,11 d_4 Cyclopropane Acid
% d_0	1.0	3.0	0.4	3.4
% d_1	3.5	4.0	1.4	1.4
% d_2	95.5	93.0	4.0	4.1
% d_3			15.1	14.7
% d_4			79.1	76.4

δ -0.36 (1H) was observed as expected. The ^{2}H NMR spectrum of the above compound (Figure 14) featured one signal at δ 0.93 (Errors of ± δ 0.5 are not uncommon when an external reference is used). Comparison of the ^{13}C NMR spectrum of the non-labelled and biosynthetic cyclopropane (Figures 15 and 16) showed that the spectrum of the latter compound lacked a signal at 16.2 ppm (previously assigned by Batchelor et al. (51) to C-9 and C-10). Also, as can be seen from Table 5, p. 54, β isotope shifts were observed for the signals of the adjacent methylenes at C-8 and C-12 (0.2 ppm) and of the bridgehead carbon atom at C-20 (0.2 ppm). On the basis of the above evidence, it was concluded that scrambling of deuterium had not occurred on biological cyclopropanation of oleic acid-9,10-d_2.

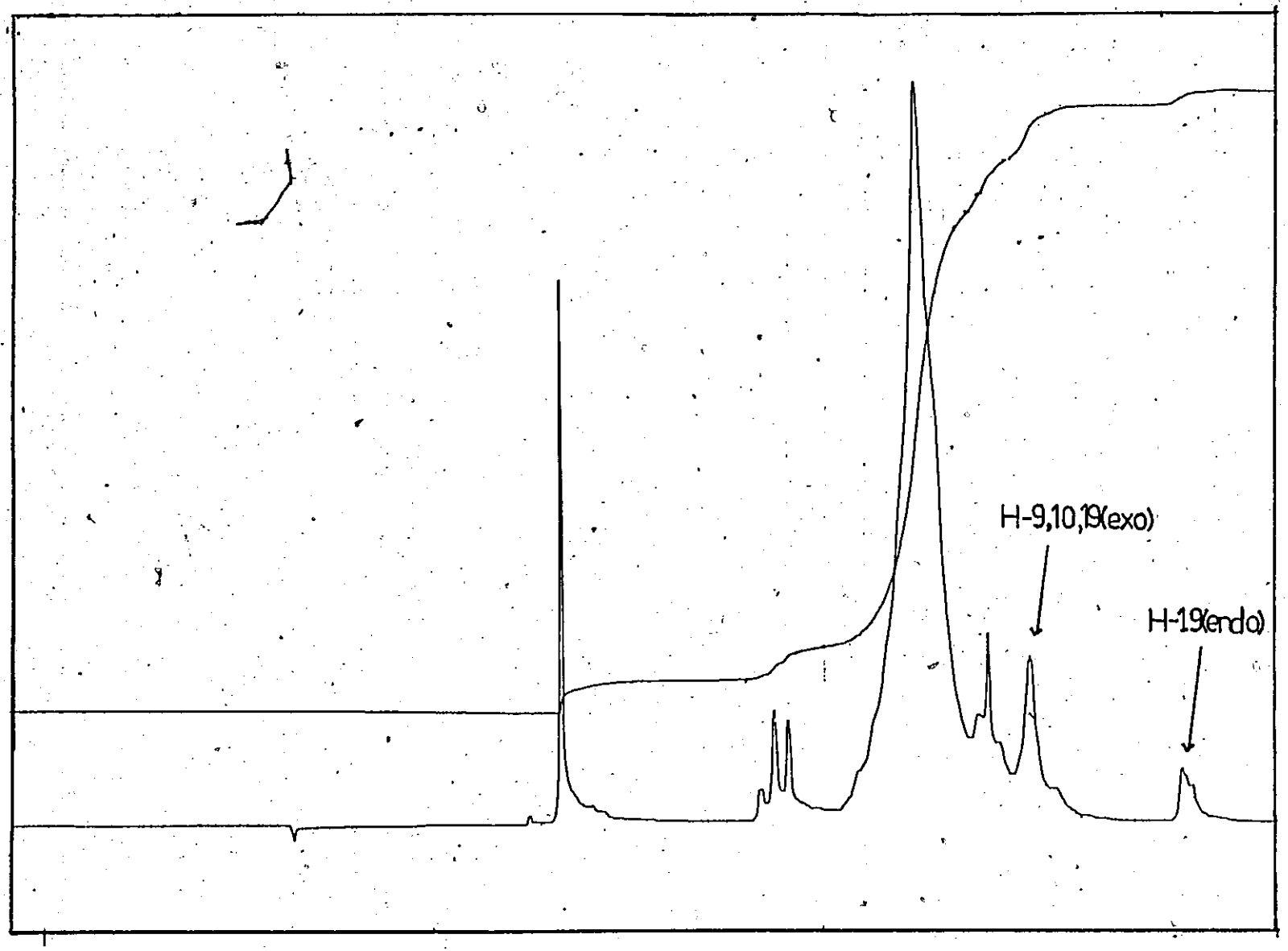


Figure 12. ^{1}H NMR spectrum of methyl dihydrosterculate.

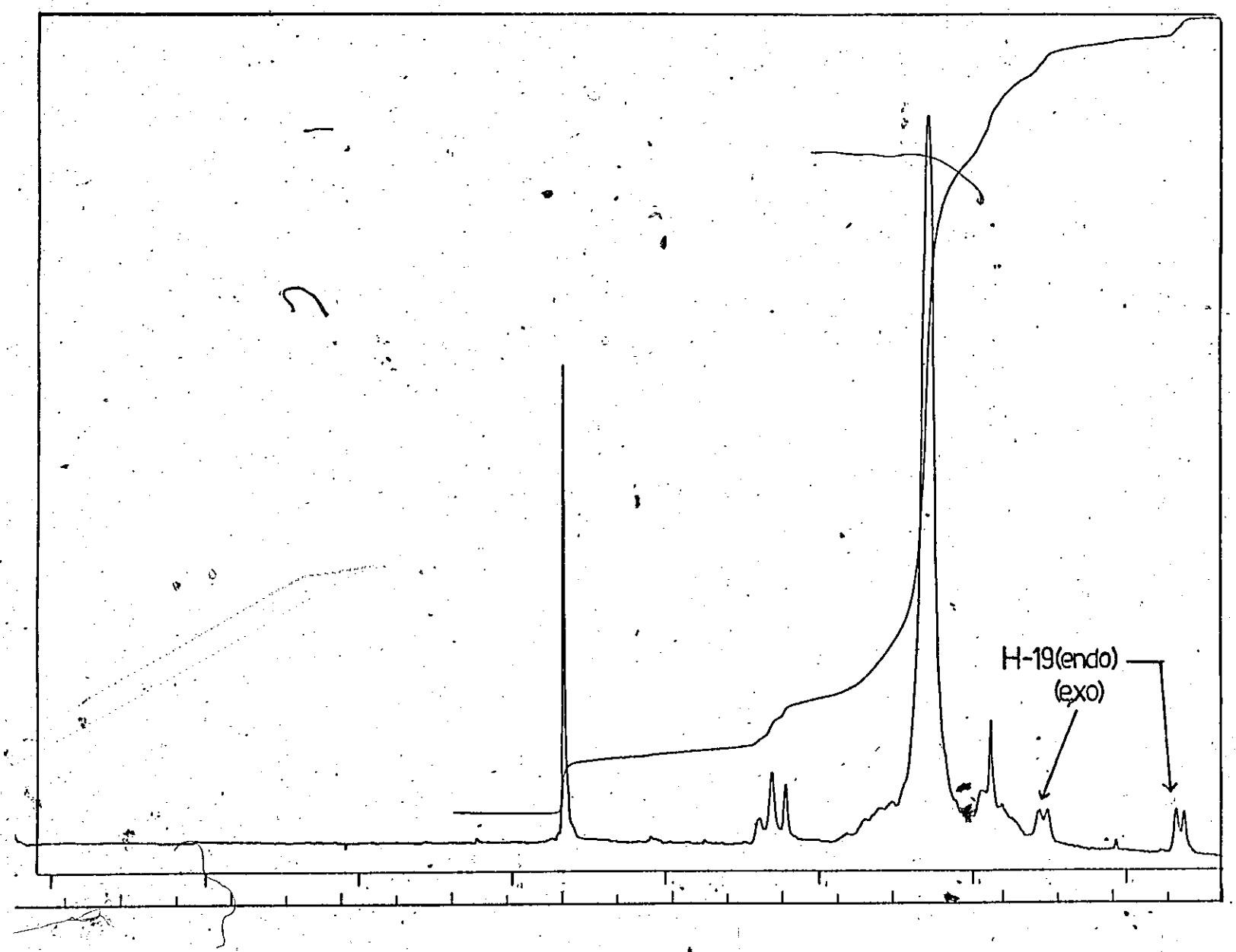


Figure 13. ^{1}H NMR spectrum of biosynthetic methyl dihydrosterculate-9,10-d_2.

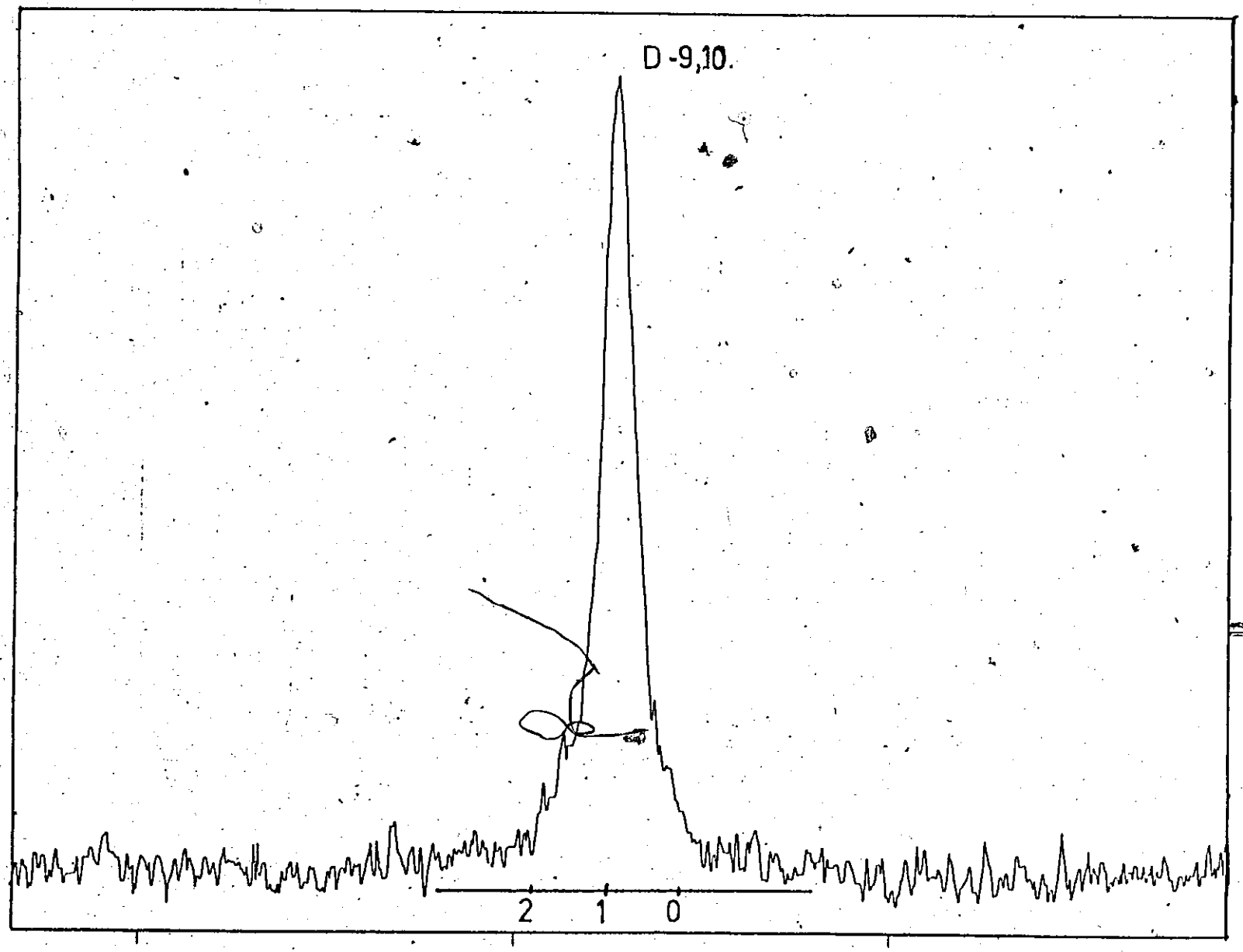


Figure 14. ^{2}H NMR spectrum of biosynthetic methyl dihydrosterculate-9,10-d_2.

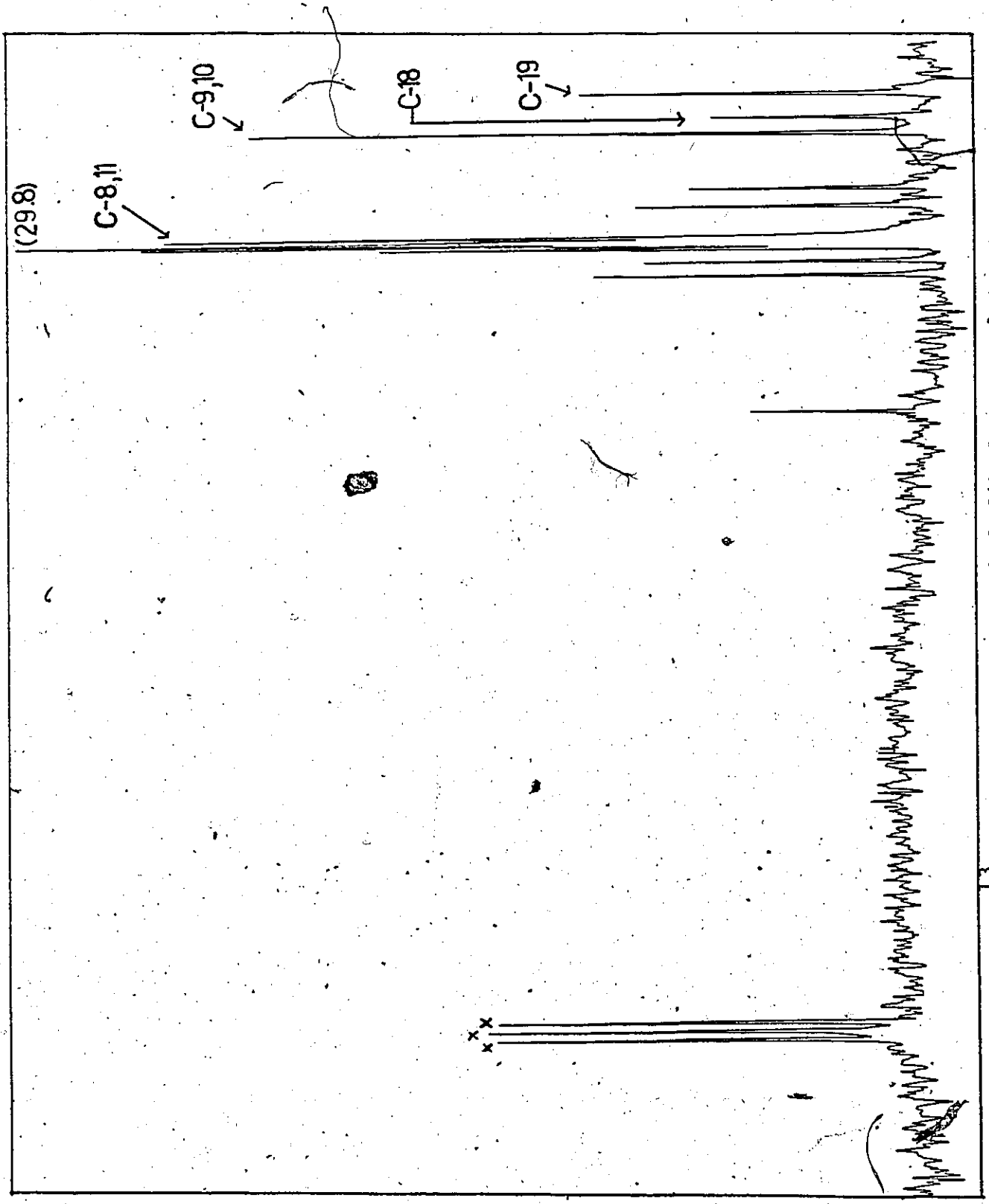


Figure 15. ^{13}C NMR spectrum of methyl dihydrosterculate.

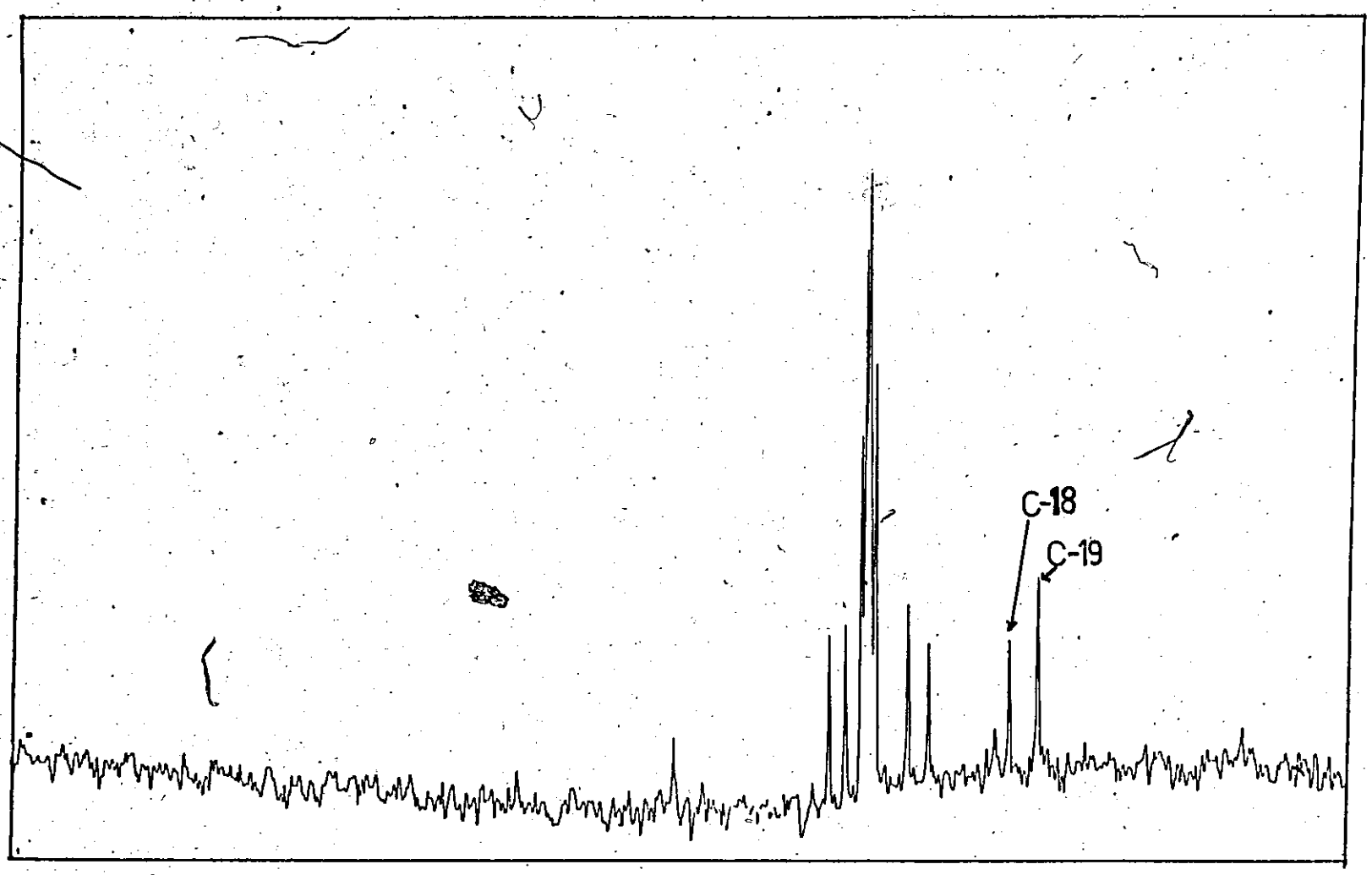


Figure 16. ^{13}C NMR spectrum of biosynthetic methyl dihydrosterculate-9,10-d_2.

That no scrambling of label had occurred in oleic acid-8,8,11,11-d_4 feeding was clear from the following spectroscopic data. The ^1H NMR spectrum of the biosynthetic cyclopropane (Figure 17) revealed that the signals of the cyclopropyl protons were not reduced in intensity and further that the multiplet at δ 0.66 was sharpened because of the absence of hydrogen at C-8 and C-11. The ^2H NMR spectrum showed one peak at δ 1.3 as expected (Figure 18). The ^{13}C NMR spectrum of the biosynthetic sample was identical with that of a synthetic sample prepared by a Simmons-Smith reaction on methyl oleate-8,8,11,11-d_4 (Figures 19 and 20).

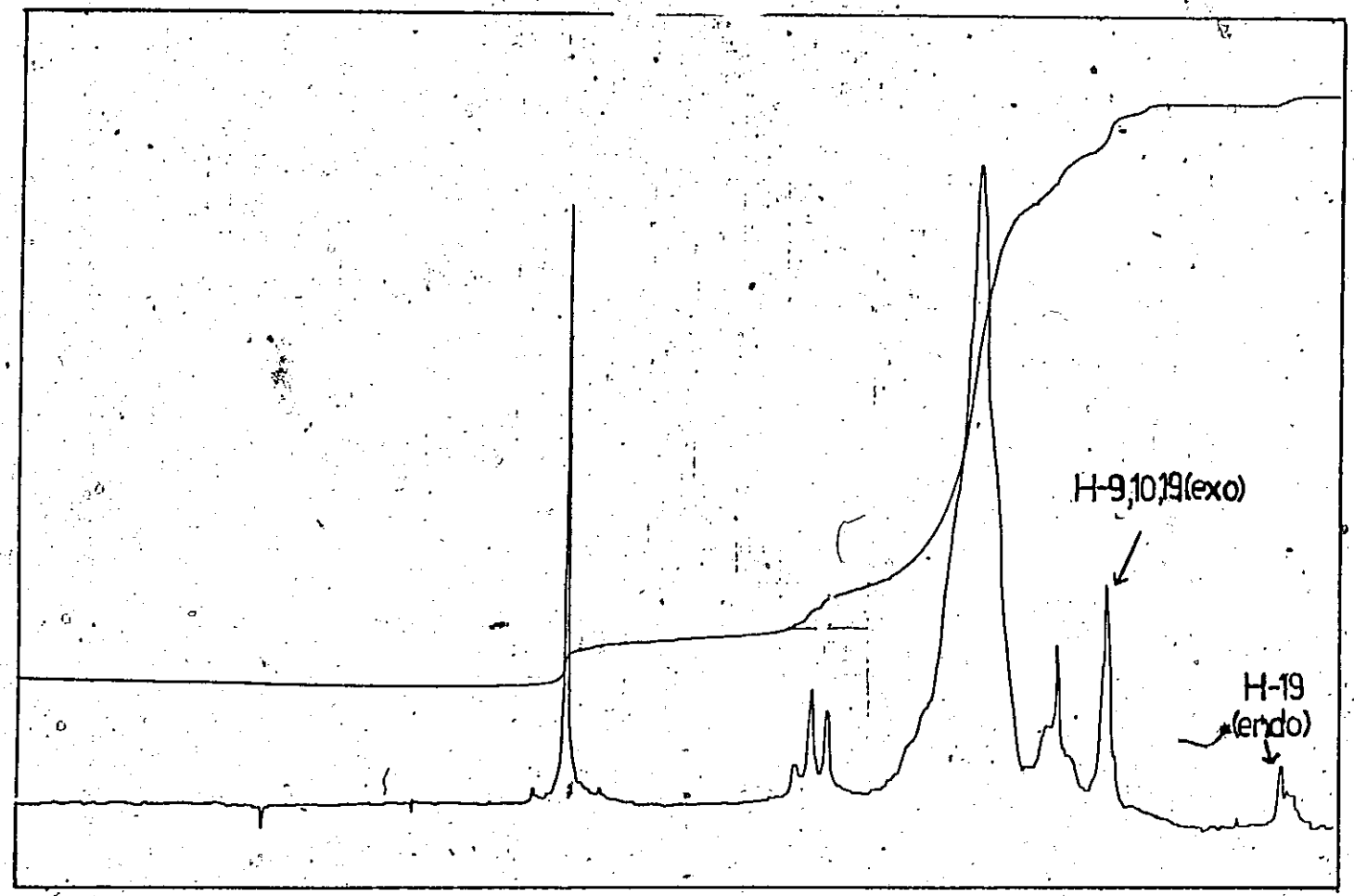


Figure 17. ^{1}H NMR spectrum of biosynthetic methyl dihydrosterculate-8,8,11,11-d_4.

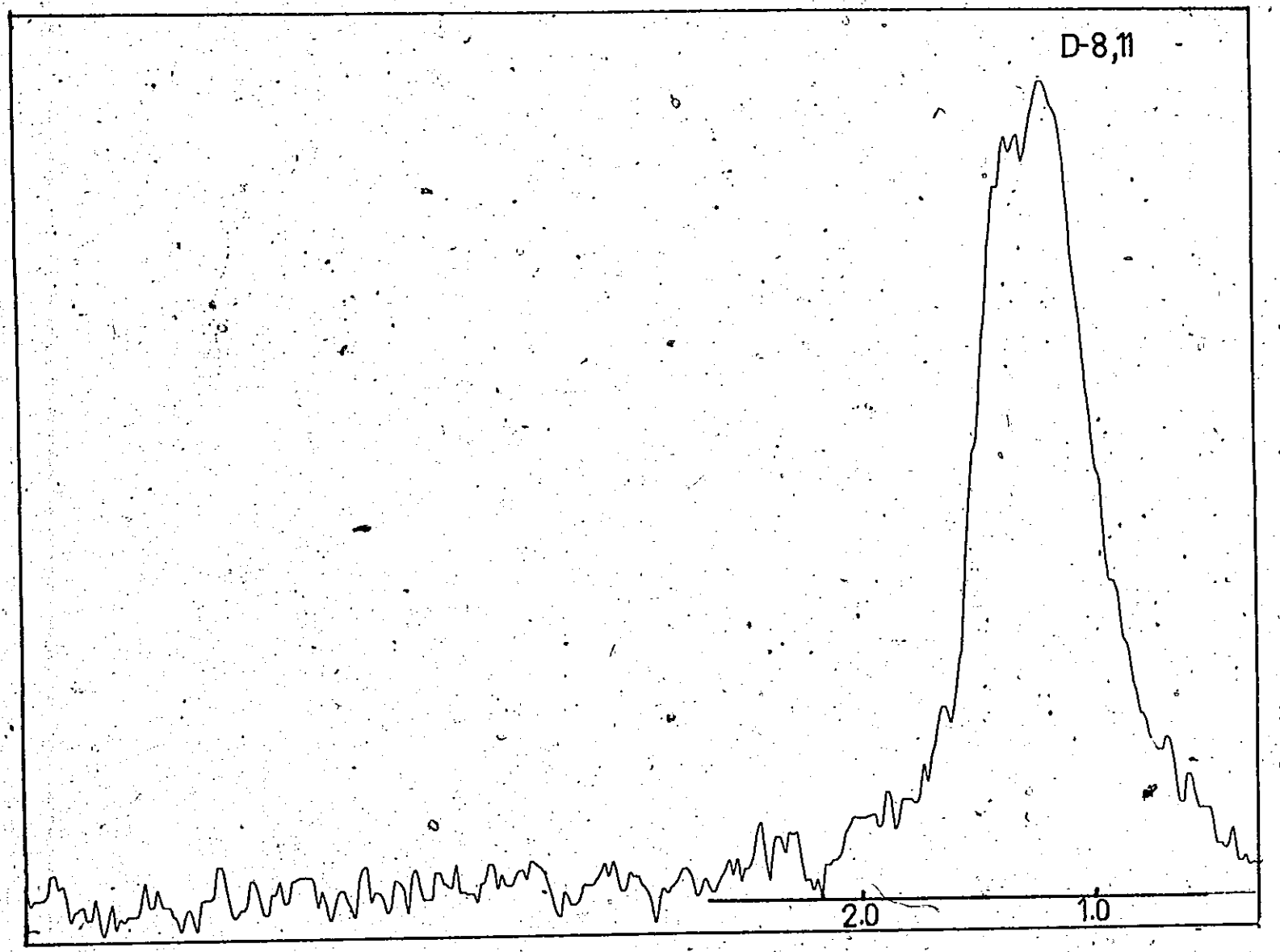


Figure 18. ^{2}H NMR spectrum of biosynthetic methyl dihydrosterculate-8,8,11,11-d_4.

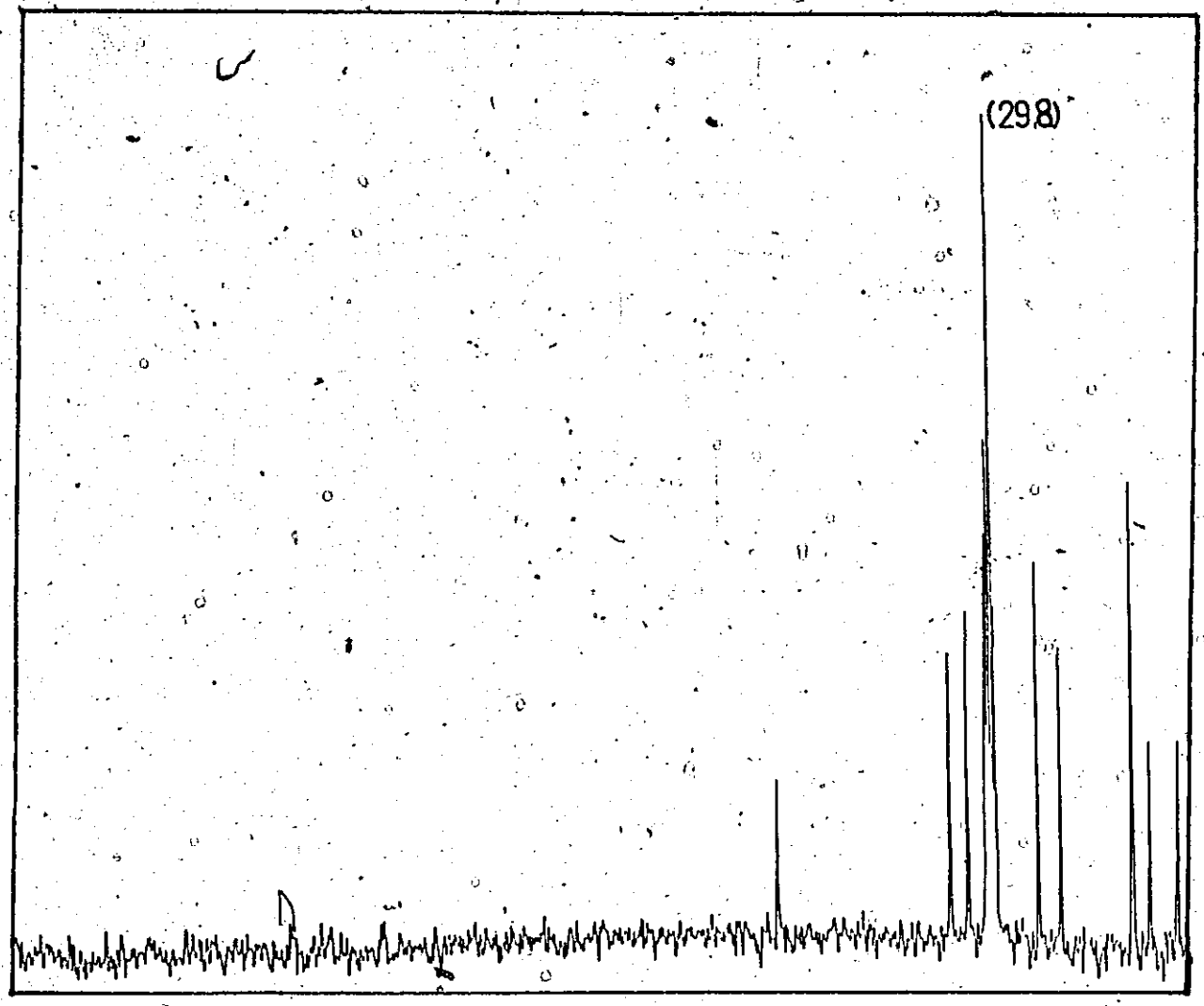


Figure 19. ^{13}C NMR spectrum of synthetic methyl dihydrosterculate-8,8,11,11-d_4.

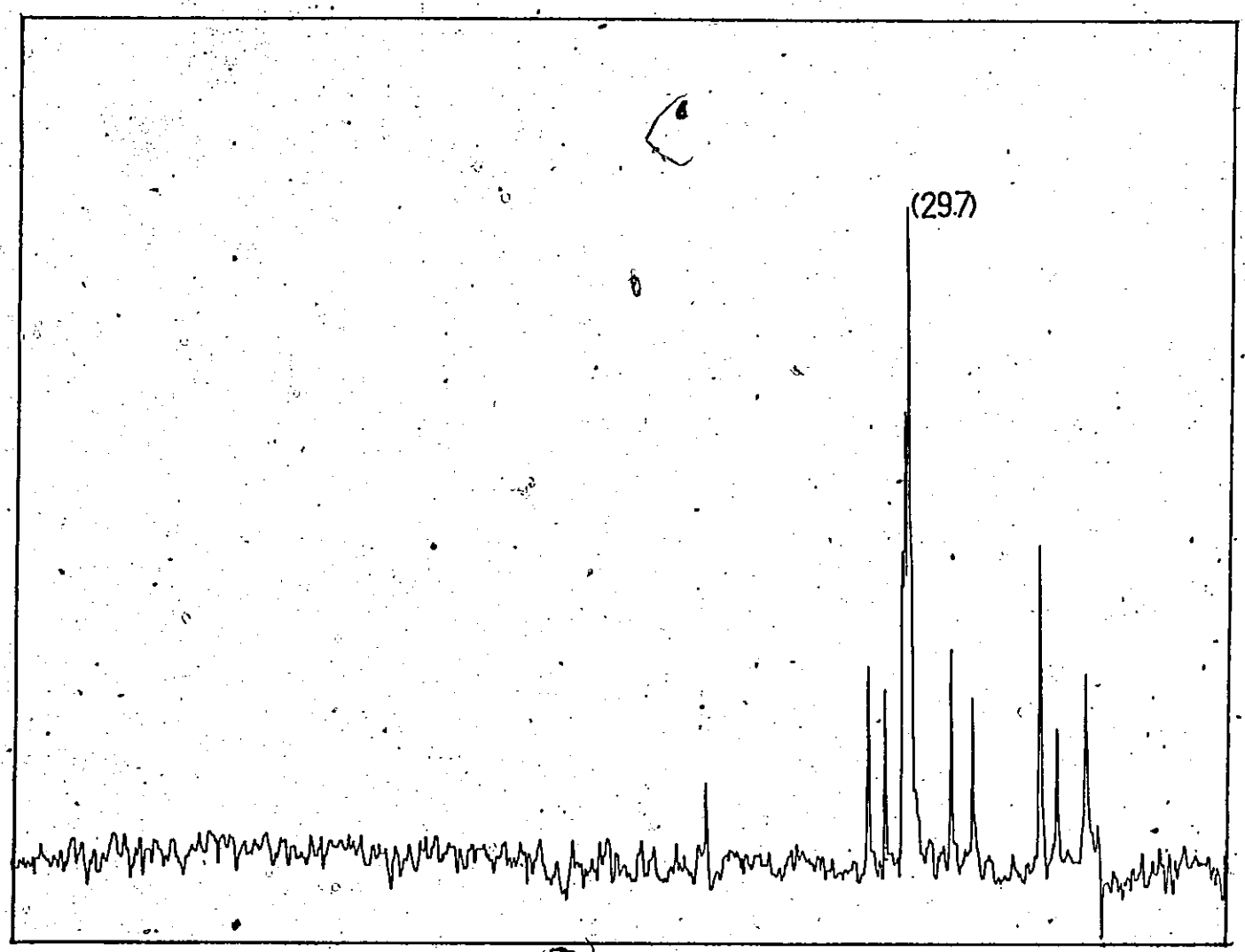
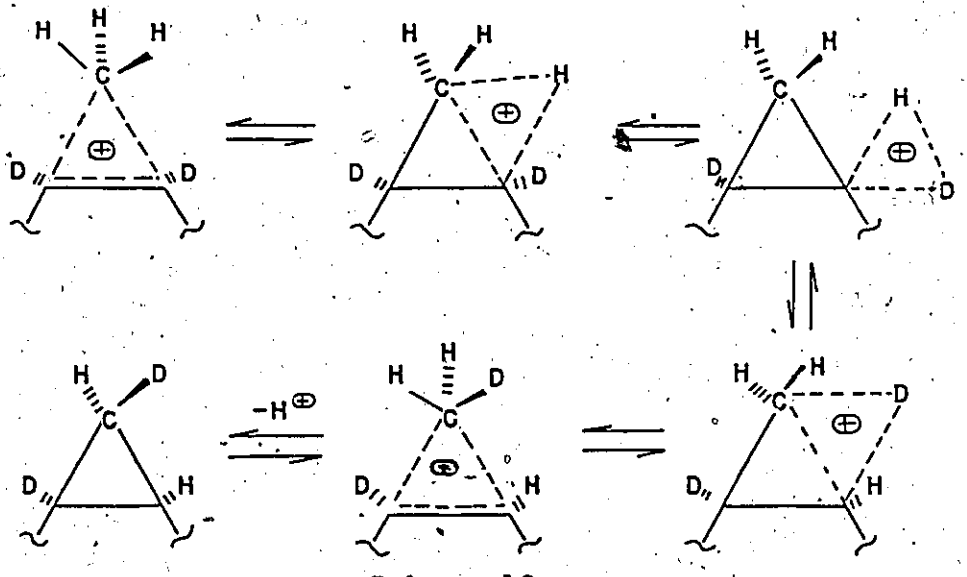


Figure 20. ^{13}C NMR spectrum of biosynthetic methyl dihydrosterculate-8,8,11,11-d_4.

The implications of the above results are the following:

1) Deuterium was not lost from the vinyl positions during the biological cyclopropanation reaction in accordance with the results of Polacheck et al. (6). The fact that deuterium scrambling did not occur means that if the carbonium ion mechanism of Scheme 1 is correct, then the protonated cyclopropane intermediate does not equilibrate between various edge or corner protonated species (52) (see Scheme 13).



Scheme 13

2) The fact that deuterium was not lost from the allylic positions during cyclopropanation conclusively rules out any mechanism where an unsaturated centre is formed at these carbon atoms (see Schemes 3-5).

3) Thus, two mechanistic possibilities for the cyclopropanation reaction remain, the carbonium ion mechanism (Scheme 1), and the carbenoid mechanism (Scheme 2). A clear choice between

these two alternatives on the basis of *in vivo* experiments would be difficult. Nevertheless, a series of experiments, designed to provide more information on the cyclopropanation reaction was carried out.

D. METHIONINE-METHYL-d_3 FEEDINGS

L-Methionine-methyl-d_3 (d_3, 95.8%; d_2, 1.4%; d_1, 0.9%; d_0, 1.9%) was fed to the micro-organism under a variety of conditions and the biosynthetic product was examined by NMR methods and by MS.

As expected, deuterium label from methionine-methyl-d_3 was found only at the bridgehead carbon atom. In the ^1H NMR spectrum, the signal present in the undeuteriated analogue at δ -0.3 was absent and there was a reduction in intensity of the signal at δ 0.66 (Figure 21). The ^2H NMR spectrum had two peaks of equal intensity at δ 0.42 and δ -0.5 (Figure 22). Curve fitting and integration showed the peak intensities to be in the ratio of 49.4 ± 2 to 50.5 ± 2, indicating that the monodeuterated species amounting to 14.5% of the total sample (*vide infra*), was equally labelled at both bridgehead positions. Inspection of the ^{13}C NMR spectrum (Figure 23) showed that the deuteron appearing at δ 0.42 was not present at C-9 or C-10 since the signal attributed to these carbons, although showing a β isotope shift of 0.2 ppm (see Table 5, p. 54) was not reduced in intensity. The signal assigned to the bridgehead carbon atom in the undeuteriated compound was absent.

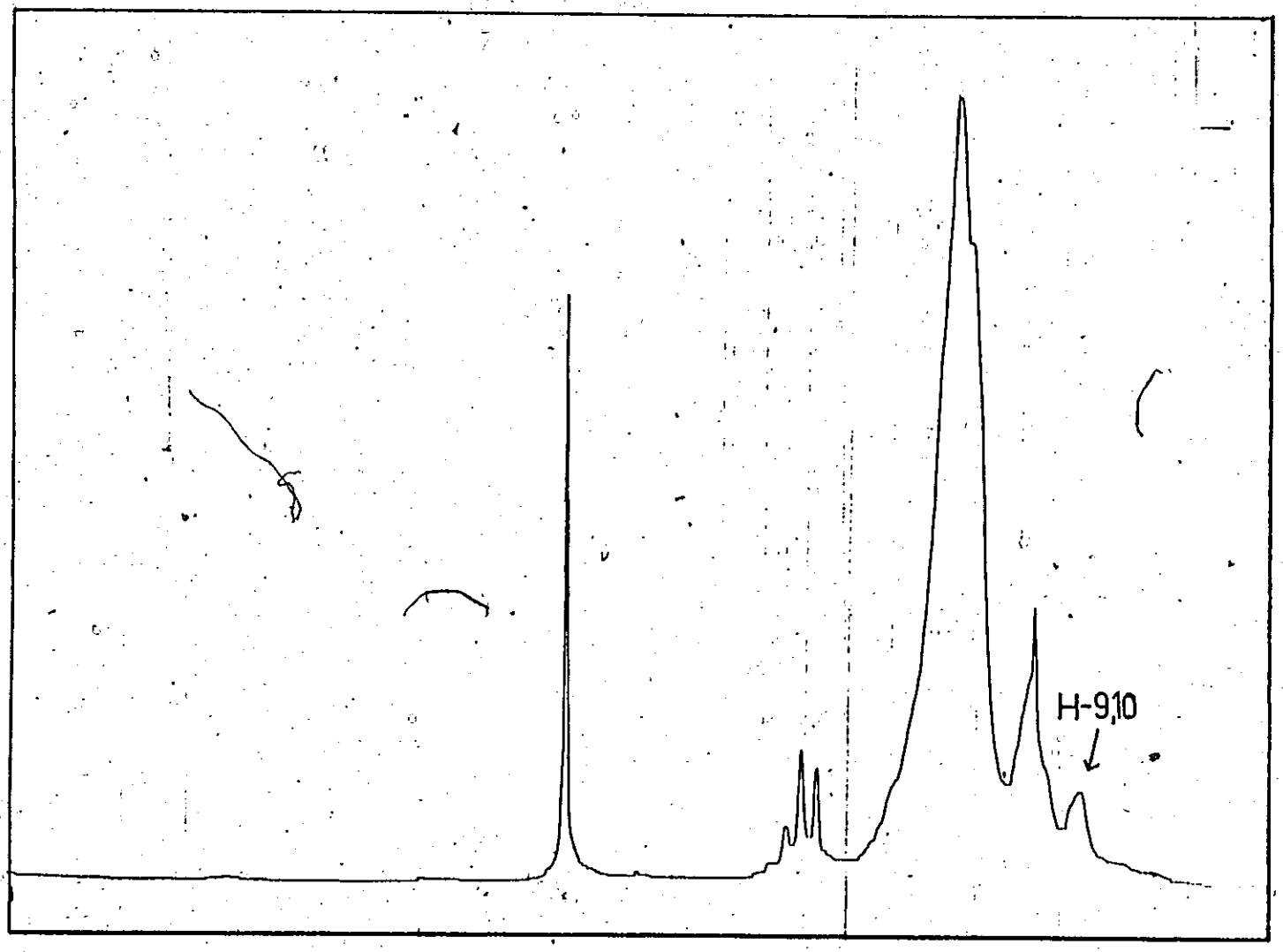


Figure 21. ^{1}H NMR spectrum of biosynthetic methyl lactobacillate-19-d_2.

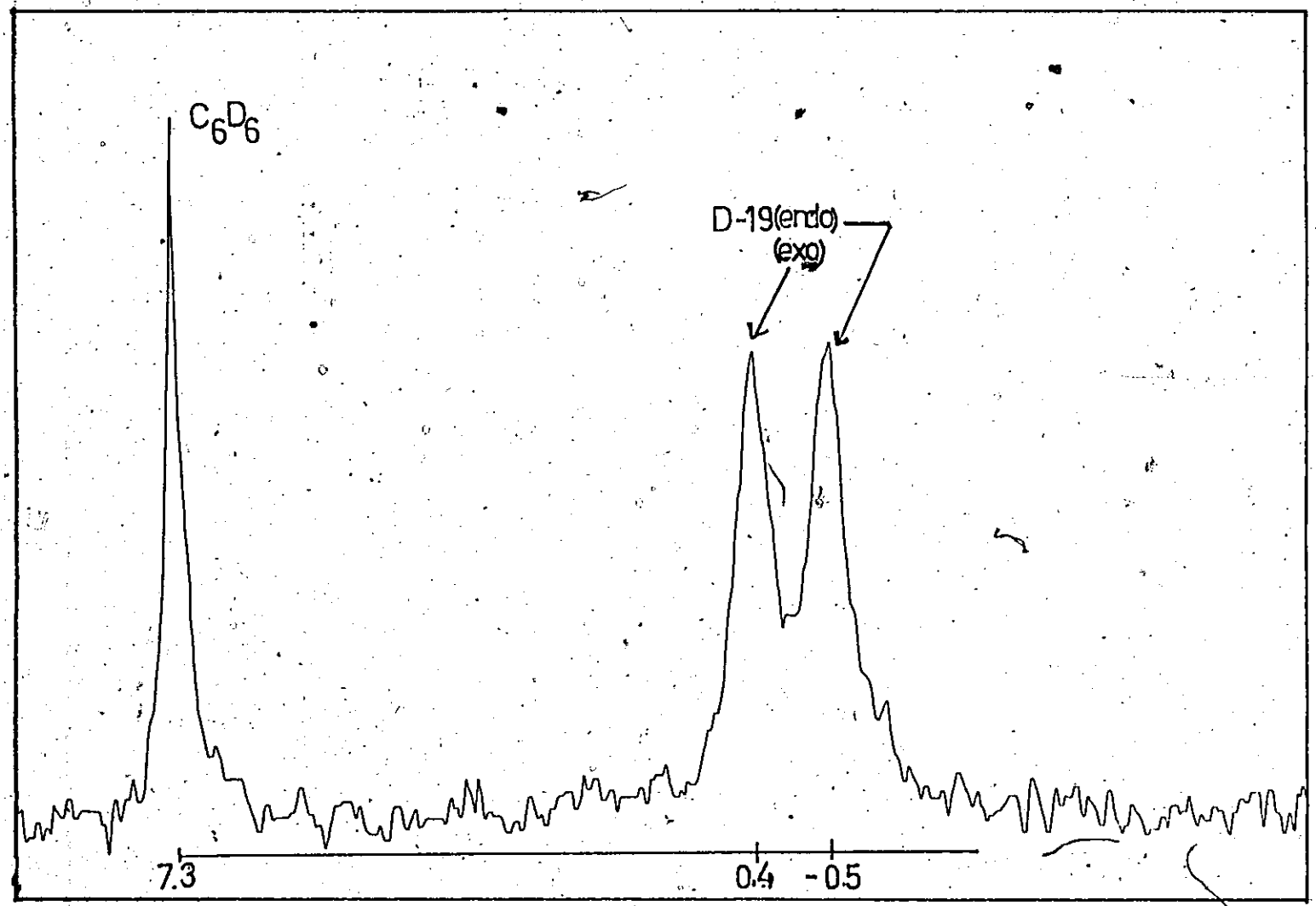


Figure 22. ^{2}H NMR spectrum of biosynthetic methyl lactobacillate-19-d_2.

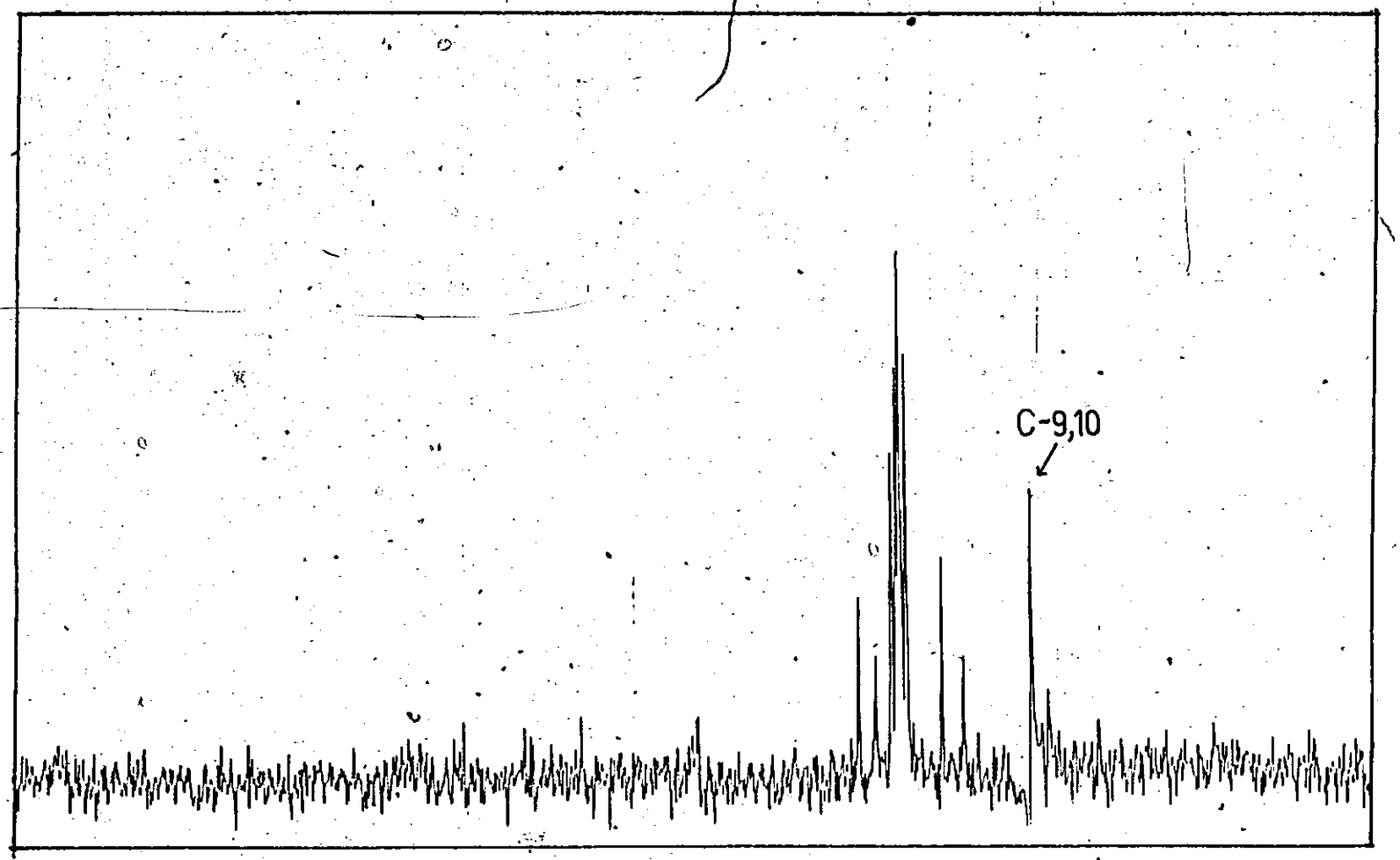
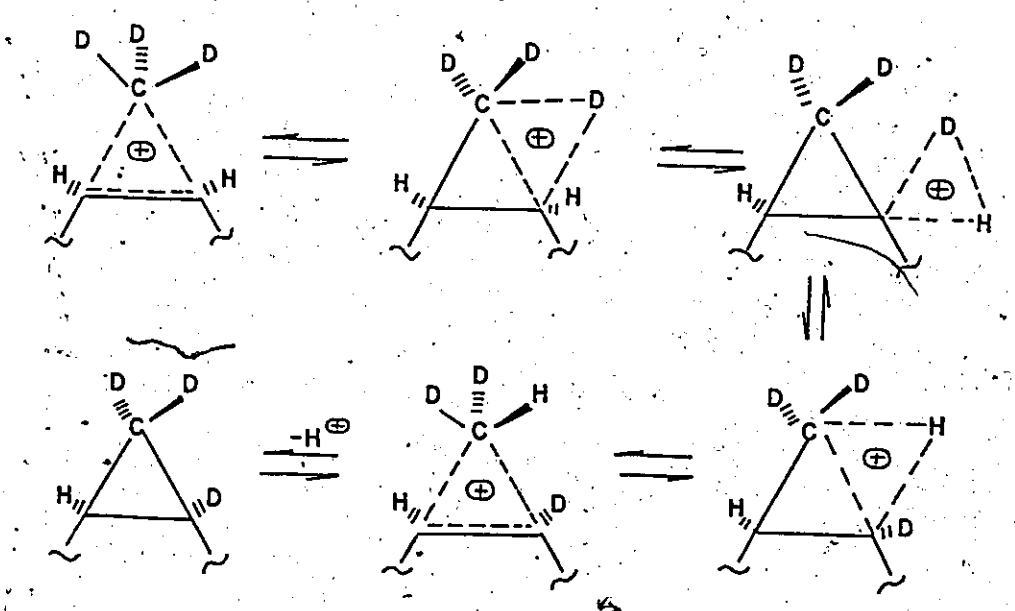


Figure 23. ^{13}C NMR spectrum of biosynthetic methyl dihydrosterculate-19-d_2.

By MS it was shown that a significant amount of d_1-cyclopropane fatty acid accompanied the expected d_2-species. The results are summarized in Table 3.

The biosynthetic cyclopropane fatty acid from Experiment h(1) was closely examined by GC/MS for the presence of a d_3-cyclopropane fatty acid. This was done by comparing the $M^+ + 1/M^+$ ratio for synthetic methyl lactobacillate-19-d_2 with that of the methyl lactobacillate derived from methionine-methyl-d_3. The results of three GC/MS runs are shown in Table 4.

A d_3-species might have arisen by the mechanism shown in Scheme 14. The fact that such a species was not found is consistent with the observation that scrambling of the label was not detected in the oleic acid-9,10-d_2 feeding experiment.



Scheme 14

Table 3

Deuterium Content of Biosynthetic Cyclopropane Fatty Acids Derived from Methionine-Methyl-d_3

Experiment*	% d_0	% d_1	% d_2	% d_1/% d_1 + % d_2
(e) (biotin) 36 hr	3.9	9.7	86.4	9.7
(f) (oleic acid)	0.8	7.3	91.9	7.4
(g) (1) (biotin)	1.5	13.8	84.7	14.1
(2) (biotin) 64 hr	1.9	13.8	84.3	14.1
(h) (1) (biotin)	4.1	16.0	79.9	16.7
(2) (oleic acid)	3.3	5.6	91.1	5.8
(3) (vaccenic acid)	5.6	7.2	87.2	7.6

* Either biotin (10 µg/L) or the fatty acid (6.06 mM) indicated was added to the basal medium along with methionine-methyl-d_3 (0.67 mM). The experiment numbers correspond to those in sub-section III in the Experimental, Section C. Harvest times were 24 h unless otherwise noted. From examination of Table 3, it can be seen that the d_1 content varies considerably from experiment to experiment, although it is always substantial.

Table 4

Search for d_3 Species in Biosynthetic Methyl Lactobacillate from Experiment h(1)

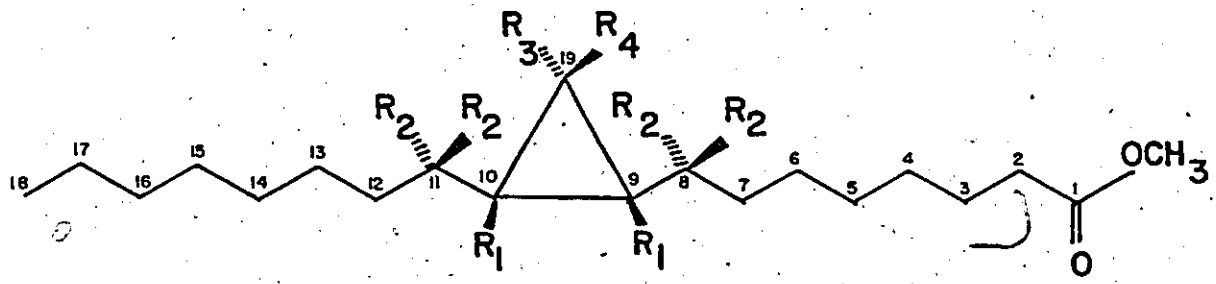
	GC/MS Run #	Synthetic Methyl Lactobacillate-19-d_2	Biosynthetic Methyl Lactobacillate-19-d_2
(M + 1)/M	1	.233 ± .01	.232 ± .01
(d_2)	2	.236 ± .01	.237 ± .01
	3	.234 ± .01	.233 ± .01
	Average	.234 ± .01	.234 ± .01
Corrected for M+1,M+2 contribution from d_1-species		.234 ± .01	.230 ± .01

Table 5

^{13}C Chemical Shifts of Deuterium Labelled and Unlabelled Cyclopropane Fatty Acids

Carbon # \ Comp'd	1	2	3	4	5
1	173.3	(-)	173.3	173.3	(-)
2	34.1	34.1	34.1	34.1	34.1
3	25.3	25.3	25.3	25.3	25.3
4	29.5	29.8	29.6	29.5	29.5
5	29.8	29.8	29.8	29.7	29.8
6	30.1	30.1	30.2	30.1	30.2
7	30.6	30.6	30.4	30.3	30.6
8	29.2	29.0	ABSENT	ABSENT	29.1
9	16.2	ABSENT	16.0	16.0	16.0
10	16.2	ABSENT	16.0	16.0	16.0
11	29.2	29.0	ABSENT	ABSENT	29.1
12	30.6	30.6	30.4	30.3	30.6
13	30.1	30.1	30.2	30.1	30.2
14	29.8	29.8	29.8	29.7	29.8
15	29.8	29.8	29.8	29.7	29.8
16	32.3	32.3	32.3	32.3	32.3
17	23.1	23.0	23.1	23.0	23.1
18	14.3	14.2	14.3	14.3	(-)
$CO_2\underline{CH}_3$	50.9	50.8	50.9	50.9	(-)
19	11.3	11.1	11.3	11.2	ABSENT

Continued.....



1. $R_1 = R_2 = R_3 = R_4 = H$ (synthetic)
2. $R_1 = D$, $R_2 = R_3 = R_4 = H$ (biosynthetic)
3. $R_1 = H$, $R_2 = D$, $R_3 = R_4 = H$ (synthetic)
4. $R_1 = H$, $R_2 = D$, $R_3 = R_4 = H$ (biosynthetic)
5. $R_1 = R_2 = H$, $R_3 = R_4 = D$ (biosynthetic)

(The ^{13}C chemical shifts of methyl-dihydrosterculate have been assigned by Batchelor et al. (51).)

E VARIATION OF EXCHANGE AS A FUNCTION OF CELL GROWTH

The fact that d_1-cyclopropane fatty acids were obtained in feedings with methionine-methyl-d_3 (see Section D), meant that at some stage in cyclopropane ring biosynthesis, a d_2 or d_1-methyl group species had been formed. Since isotope effect studies, using methionine, deuteriated at the methyl group, were planned, an attempt was made to establish conditions where loss of deuterium via exchange processes would be minimized.

It had previously been observed (Table 3) that adding either cis-vaccenic or oleic acid in lieu of biotin to the medium, reduced the extent of d_1-species formation markedly. It was also noted that growth on the fatty acid containing medium was generally poor (see Experimental, Section C.III, (h).). This observation led to the hypothesis that the extent of exchange increased with cell growth. A systematic study of cyclopropane fatty acid biosynthesis and extent of exchange as a function of cell growth was carried out (see Figure 24). A wide variation in the rate of cyclopropanation was noted, even when cells were grown on the same medium (cf. Curves B and C). In general, however, it can be seen that the percentage of cyclopropane fatty acids increases with culture age as has been noted by other workers (53). The results of the large scale incubation, (Curve C) where the cells were harvested at the points indicated on Figure 24, are presented in Table 6.

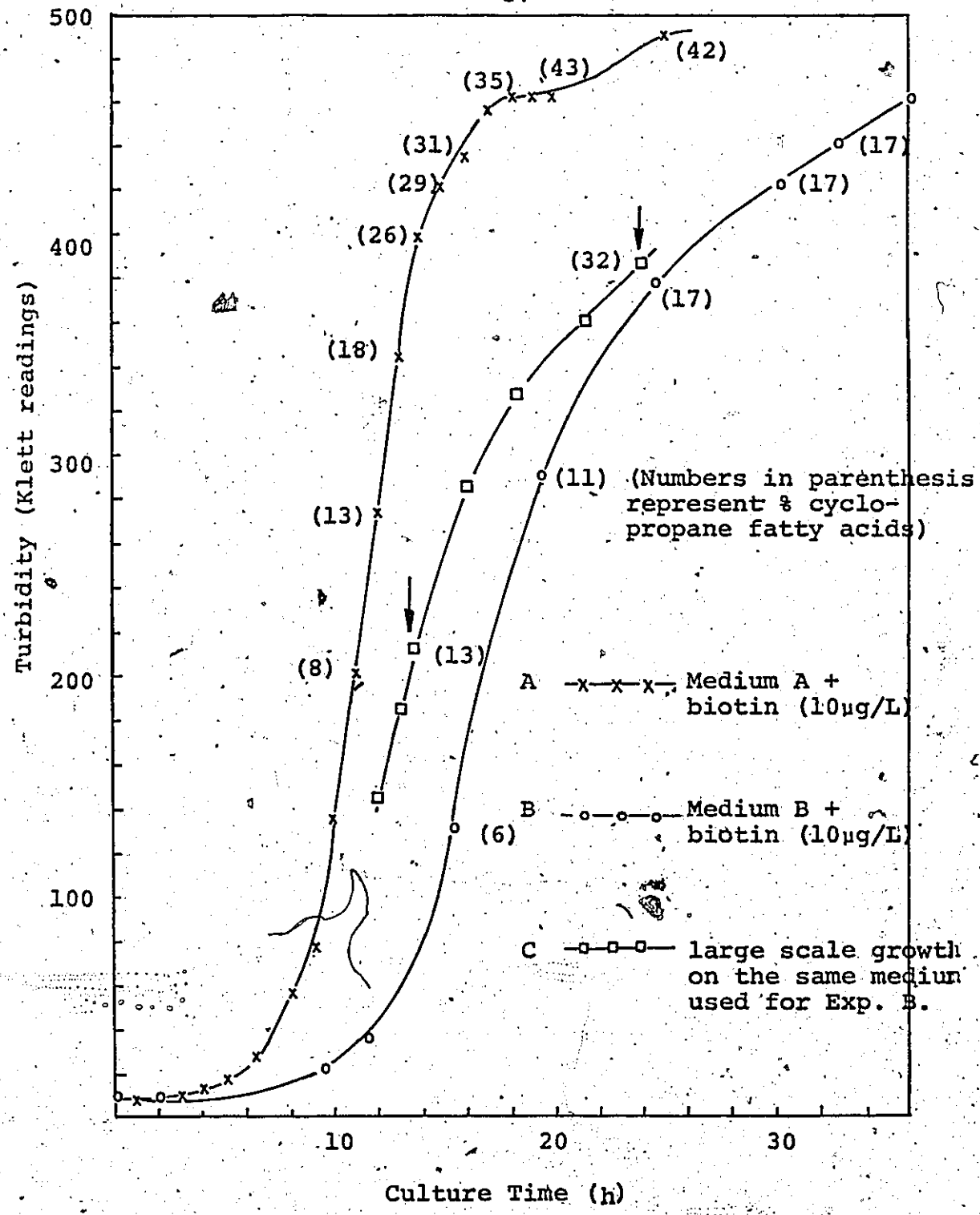


Figure.24. Cyclopropane Fatty Acid Biosynthesis versus Cell Growth.

Table 6.

Analytical Data obtained for Cells Isolated at 13.5 and 24 h

	Cells isolated at 13.5 h	Cells isolated at 24 h
pH of medium at time of isolation	5.9	4.4
Ave. Turbidity at time of isolation	210	401
Cell Yield (mg/100 mL of culture)	52	124
Fatty Acid conteht (mg)	4.7	20.7
% Cyclopropane Fatty Acid	13	32
Deuterium content of cyclopropane fatty acid		
% d_0	8.8	4.4
% d_1	7.3	11.3
% d_2	83.9	84.3
% d_1/% d_1 + % d_2	8.0	11.8

As was predicted, the d_1-content of the cyclopropane fatty acids in the cells isolated at early exponential phase (13.5 h) was substantially (50%) lower than that of the cyclopropane fatty acids in the cells isolated at late exponential phase (24 h). One factor which might control the extent of exchange is the pH of the medium which drops at a rate proportional to cell growth. This hypothesis was not tested experimentally.

It did not appear feasible to eliminate the exchange process entirely since even at early exponential phase, the amount of d_1-cyclopropane fatty acid was sizable (8.0%). As will be seen in Section F, this fact introduced some degree of uncertainty into the intramolecular isotope-effect measurements.

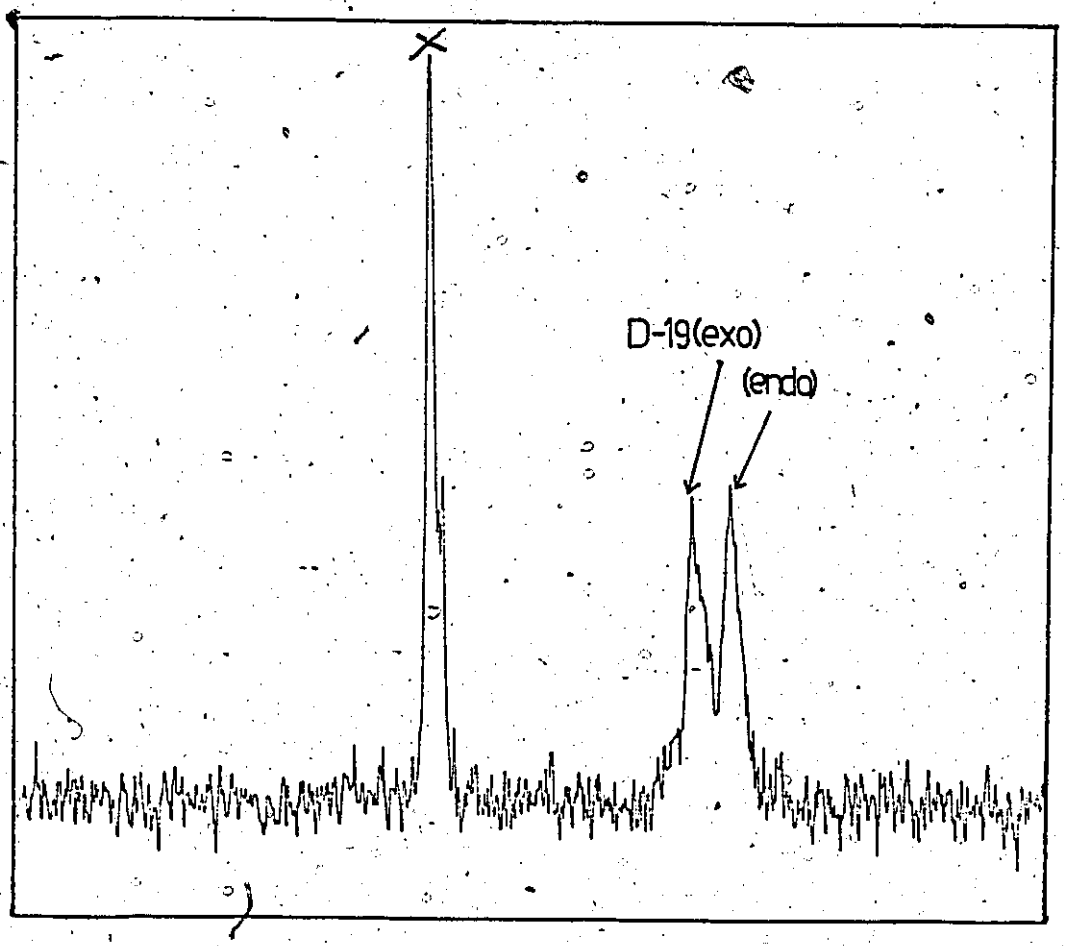


Figure 25. ^{2}H NMR Spectrum of methyl lactobacillate-19-d_1 biosynthesized from methionine-methyl-d_1.

F PRIMARY DEUTERIUM ISOTOPE EFFECT STUDIES

Attention was turned to primary deuterium isotope effect

studies as a means of investigating the mechanism of the biological cyclopropanation reaction. Because of the torsiosymmetry of the methyl group, both an intra- and intermolecular isotope effect for the proton abstraction step in the cyclopropanation reaction, could be measured. (All hydrogens or isotopes of hydrogen on a methyl group are equivalent to one another by virtue of rotation. ^2H NMR of a d_1-cyclopropane fatty acid derived from methionine-methyl-d_1 showed that the lone deuterium occupied both bridgehead positions to the same extent (see Figure 25). Thus, there had been no discrimination between the two prochiral hydrogens on a d_1-methyl group species.)

(I) Intermolecular Isotope Effect Study

A 1:1 mixture of non-deuteriated L-methionine and L-methionine-methyl-d_3 was fed to L. plantarum and the distribution in the resulting biosynthetic cyclopropane fatty acid measured to be: d_0, 51.6%; d_1, 5.9%; d_2, 42.5%. An intermolecular primary deuterium isotope effect, k_H/k_D, was calculated from the following ratio: $51.6 \pm 1/(5.9 \pm 1 + 42.6 \pm 1) = 1.07 \pm 0.04$. The lack of any significant intermolecular isotope effect means that proton-carbon bond cleavage is not a rate-determining step in the cyclopropanation reaction. (This interpretation assumes that biological cyclopropanation does not deplete the cell of S.A.M., in which case, no isotope effect would be observed. This assumption is probably correct,

see Introduction, p. 12.)

It is tempting to account for the negligible intermolecular isotope effect in terms of a carbonium ion mechanism (Scheme 1) where deprotonation of a carbonium ion intermediate would be expected to be a more facile process than methyl transfer to an unactivated double bond. On the other hand, in the carbenoid mechanism (Scheme 2), formation of the non-stabilized sulfur ylid would probably be slower than carbenoid transfer and subsequent methylene transfer. This type of reasoning has its limitations, since as Cleland has pointed out (54), substrate binding, conformational changes, product release, etc., are often the rate-limiting steps in an enzyme-catalyzed reaction. If this were, in fact, so, then an intermolecular isotope effect on the deprotonation step would be obscured kinetically. The situation would be greatly clarified if one could demonstrate that an intermolecular isotope effect was operating in the carbon-carbon bond formation step of the cyclopropanation reaction.

The possibility that carbon-proton bond cleavage was, in fact, rate-limiting but the isotope effect intrinsically low for other mechanistic reasons (55) was ruled out by measuring the intramolecular deuterium isotope effect; when a methyl group species, which is mono- or di-deuterated, is used for this purpose, an intrinsic isotope effect is obtained, which is independent of the degree to which proton (deuteron) abstraction from the methyl group is rate-limiting. This is

because the reagent must "make a choice" between otherwise equivalent ligands and this choice will be directly related to k_H/k_D. It must be emphasized that this situation is only found for reactions at a group where two or more ligands are made equivalent by rotation with respect to a reagent poised to remove one of these ligands.

(II) Intramolecular Isotope Effect Studies

Three feeding experiments were carried out simultaneously to allow better comparison of the data (c.f. Section G). The results are recorded in Table 7.

Since the extent of exchange at the methyl group, in the methionine-methyl-d_1 and methionine-methyl-d_2 feedings could not be determined, only a minimum intramolecular isotope effect was calculated. This value was calculated to be $k_H/k_D = 2(\%\ d_2/\%\ d_1) = 2(63.8 \pm 2/35.1 \pm 2) = 3.6 \pm 0.2$, for the methionine-methyl-d_2 feeding and $k_H/k_D = \frac{1}{2}(\%\ d_1/\%\ d_0) = \frac{1}{2}(86.5 \pm 2/13.5 \pm 2) = 3.2 \pm 0.5$, for the methionine-methyl-d_1 feeding. (See Appendix IIA for details of the calculation.) Exchange in the methionine-methyl-d_1 feeding would be less than in the methionine-methyl-d_2 feeding and thus the calculated k_H/k_D from the former experiment would be more accurate. This value is, therefore, used in the following discussion. From the experiments of Pohl et al. (5), it is possible to extract an intramolecular tritium isotope effect of 5.3 for the biological cyclopropanation reaction (see

Table 7. Results of Intramolecular Isotope Effect Experiments

Compound Administered (length of incubation, h)	Compound Isolated	pH at End of Incubation	Cell Yield (mg/100 mL of culture)	% d_0	% d_1	% d_2	% d_1
methionine-methyl-d_3				13.8	0	1.2	85.0
(29)	cyclopropane fatty acid	5.0	106	16.2	10	73.8	
				(0.8)	(11.5)	(87.7)	
methionine-methyl-d_2				0.6	2.8	96.6	
(29)	cyclopropane fatty acid (I)	4.8	110	4.1	35.6	60.3	
				(1.1)	(35.1)	(63.8)	
(48)	cyclopropane fatty acid (II)	4.5	120	3.7	37.7	58.7	
methionine-methyl-d_1				3.0	97.0		
(29)	cyclopropane fatty acid (I)	5.2	111	17.8	82.2		
				(13.5)	(86.5)		
(48)	cyclopropane fatty acid (II)	4.6	122	17.7	82.3		

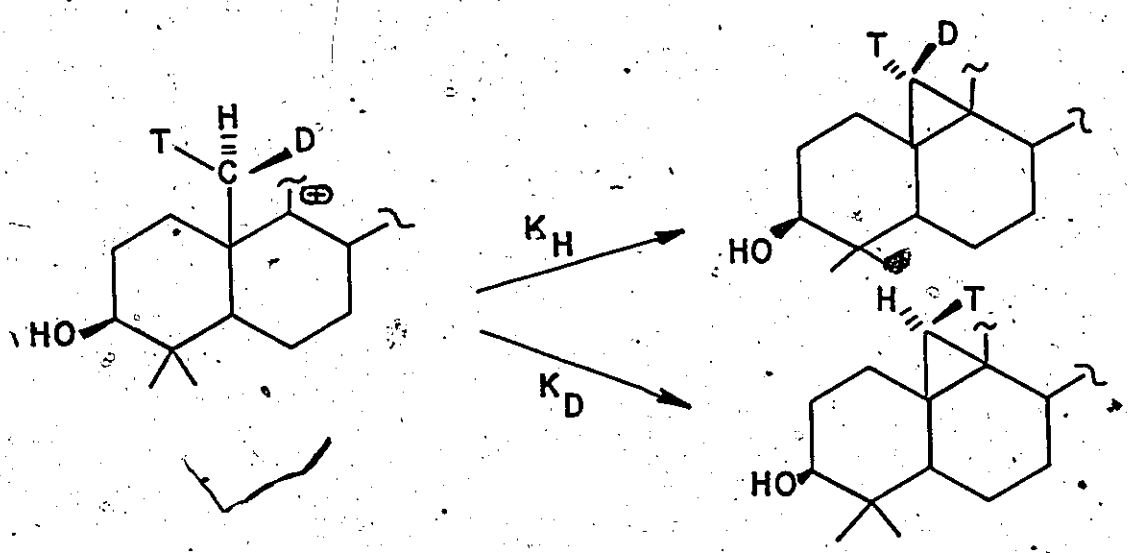
Notes to Table 7:

1) The results of Experiment II are included to show that even though these cells grew out slower than those in Exp. I, the resulting distribution of deuterium in the cyclopropyl product was similar. The results of Exp. I are used for the calculations.

2) Isotopic compositions of the cyclopropane fatty acid were corrected for both the isotopic composition of the labelled methionine (which in all cases did not consist of one isotopic species) and for endogenous d_0-methionine and d_0-cyclopropane fatty acid in the inoculum. The latter value was estimated to contribute ca. 2% to the d_0 content of the cyclopropyl product. The corrected isotopic compositions are given in parentheses to an accuracy of ± 2%.

Appendix IIB). Applying the Swain relationship (56) to the k_H/k_D obtained in the methionine-methyl-d_1 experiment, gives an intramolecular tritium isotope effect of $(3.2 \pm 0.5)^{1.44} = 5.4 \pm 0.4$, in good agreement with that obtained above.

An enzymic reaction which has been taken as the model for biological cyclopropane formation is the 1,3-elimination of a hydrogen to form cycloartenol (57). The stereochemistry of this reaction has been studied using chiral methyl-labelled oxidosqualene containing 30% tritium enrichment to allow examination of the product by ^3H nmr (58) (see Scheme 15).



Scheme 15

From the intensities of the tritium nmr signals corresponding to the two tritiated products, it was possible to calculate an intramolecular deuterium isotope effect of 2.8 ± 0.2 which compares reasonably closely to the value of

3.2 ± 0.5 obtained from the methionine-methyl-d_1 feeding (see Appendix IIC for the method of calculation).

It should be emphasized that the above correlation between intramolecular isotope effects does not by itself rule out the carbenoid mechanism. Unfortunately, an intramolecular isotope effect for biological sulfur ylid formation is not available for comparison purposes.

G QUANTIFICATION OF THE EXCHANGE PROCESS IN METHIONINE-METHYL-d_3 FEEDINGS

The extent of exchange at the methyl group in the methionine-methyl-d_3 feedings could be calculated in the following manner:

In the feeding using methionine-methyl-d_3 (Table 7, hereafter referred to as exp. A), a d_3-methyl group must have exchanged to give a d_2-methyl group which in turn might have exchanged to give a d_1 and eventually a d_0-methyl group species. In the feeding using methionine-methyl-d_2 (Table 7, hereafter referred to as exp. B), a d_2-methyl group might have exchanged to give a d_1 and eventually a d_0-methyl group species. Assuming, for the moment, that the extent to which a d_2-methyl group exchanged was the same in both experiments A and B, then one can calculate the amount of cyclopropyl product derived directly or indirectly from such a species in exp. A. Thus, associated with the 11.5% of d_1-product obtained in exp. A was a certain amount of d_2-product which also arose

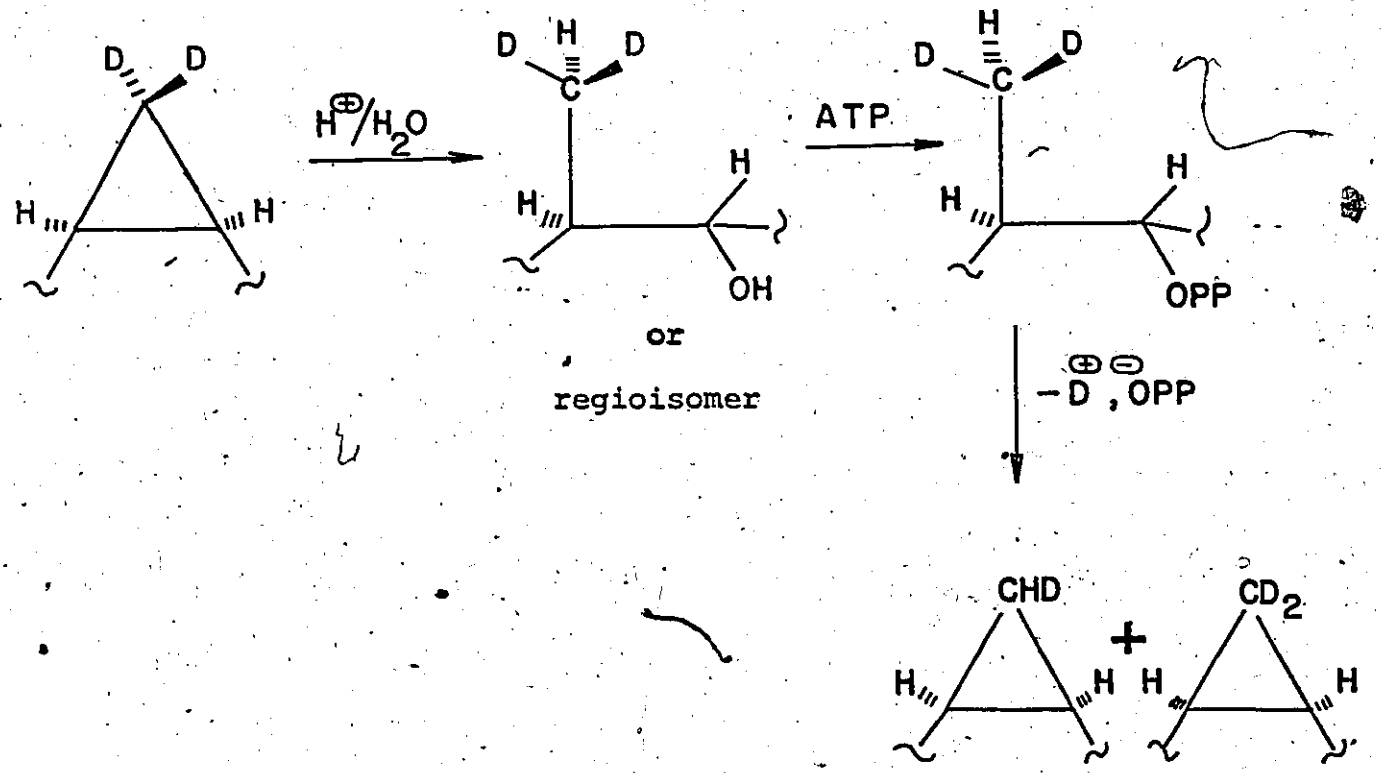
from a d_2-methyl species. This amount can be calculated by taking the ratio of the d_1 content obtained in experiments A and B and multiplying this value by the d_2 content obtained in exp. B. This is (11.5/35.1 x 63.8) = 20.9. Adding this number to the d_1 and d_0 content obtained in exp. A, gives the total amount of cyclopropane fatty acid derived from an exchanged methyl group species. The extent of this exchange was (20.9 + 11.5 + 0.8) = 33.2%.

However, the extent of exchange of a d_2-methyl group in exp. B may have been greater (never less) than in exp. A. The ratio of % d_2/% d_1 used in the above calculation might, therefore, be too low and thus the above calculated value for the extent of exchange is a minimum value. Because of the magnitude of the exchange phenomenon and the fact that similar amounts of exchanged product had been observed before in the C-alkylation of isolated double bonds (see section H, VII), it was considered appropriate to investigate the origin of the d_1-species in the biosynthesis of the cyclopropane ring.

H THE ORIGIN OF d_1-CYCLOPROPANE FATTY ACIDS IN FEEDINGS USING METHIONINE-METHYL-d_3

A number of possible mechanisms for the production of d_1-cyclopropane fatty acids from exchanged methyl groups was subjected to experimental test. These are listed below along with a discussion of the experimental results.

(I) The cyclopropyl fatty acids might undergo hydrolytic ring fission followed by ring closure in a series of separate enzymic reactions (see Scheme 16).



Scheme 16

To test the above proposal, the two non-labelled alcohols corresponding to those shown above, were synthesized by treatment of methyl cis-9,10-epoxyoctadecanoate with $CuLi(Me)_2$. The two regioisomers were separated by HPLC; identified by their MS, hydrolyzed to the corresponding carboxylic acids, and administered to L. plantarum in lieu of biotin. Neither compound supported growth. If conversion

to dihydrosterculic acid had occurred, growth should have resulted, since the organism will grow on this acid in lieu of biotin. This experiment does not conclusively rule out proposal (I), since the two alcohols may not have been transported into the cell, thus preventing metabolism of these compounds.

In a separate experiment, the fatty acid fraction from biotin-grown cells was checked for the presence of polar intermediates such as the above hydroxylated fatty acids. This was done by the comparison of the capillary GC chromatogram of an unsilylated fatty acid extract with that of a silylated extract. No significant difference between the two traces was noted. The search for intermediates related to cyclopropane fatty acids was not pursued further.

(II) A proposal closely related to the suggestion presented above is that the cyclopropyl compounds could re-enter the active site of cyclopropane synthetase and undergo reversible ring-opening. This idea was tested by administering lactobacillic acid-19-d_2, along with biotin to L. plantarum. (Biotin was included in the medium to allow de novo olefinic fatty acid biosynthesis to occur, which in turn might induce cyclopropane synthetase production.) As can be seen from Table 8, the d_1-content of reisolated lactobacillic acid was the same as that of the material which was fed.

Table 8

Deuterium content of Lactobacillic Acid-19-d_2 incorporated into L. plantarum

	Compound Administered	Compound Reisolated
% d_0	0	9
% d_1	2	2
% d_2	98	89

The presence of 9% of d_0-product showed that the addition of synthetic lactobacillic acid did not completely inhibit cyclopropane synthetase.

These results do not support proposals (I) or (II) as explanations for d_1-cyclopropane fatty acid production.

(III) The results do not rule out a third possibility, namely that the bridgehead d_2-methylene group might undergo reversible ring-opening while the cyclopropyl compound is still on the active site of the enzyme. (This idea will be given further consideration on p. 78.)

(IV) Another possible pathway for d_1-cyclopropane fatty acid formation is via the intermediacy of methionine-methyl-d_2. Degradation of a d_3-methyl group would lead to formaldehyde-d_2 formation, which might be used to generate methionine-methyl-d_2. Subsequent biosynthesis of S.A.M.-methyl-d_2 would then lead to a mixture of d_2 and d_1-cyclopropane fatty acids. Several pathways for the oxidative degradation of the

methyl group of methionine are discussed below (see Scheme 17).

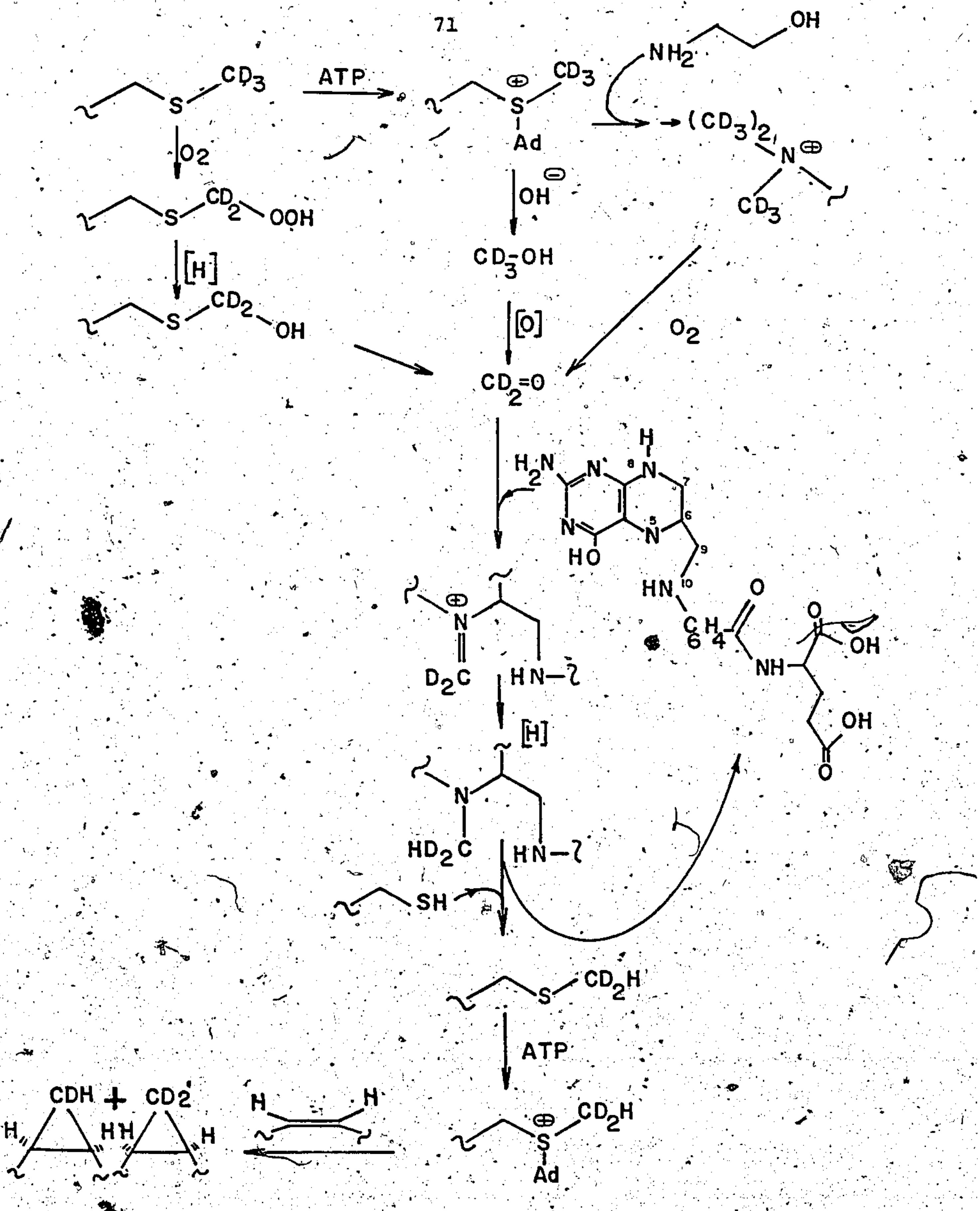
(a) Peroxidation of the d_3-methyl group of methionine, followed by reduction of the hydroperoxy group would generate a hemithioacetal which would be in equilibrium with formaldehyde-d_2.

(b) S.A.M.-methyl-d_3 might be hydrolyzed to form methanol-d_3 which, on further oxidation, would give formaldehyde-d_2.

(c) Oxidation of a methylated metabolite derived from S.A.M.-methyl-d_3, such as choline (59) would also generate formaldehyde-d_2.

A series of experiments, designed to test for the operation of the various pathways outlined above, was undertaken. Two methionine-methyl-d_3 feedings, one under anaerobic and one under aerobic conditions, were carried out (Experiments 1 and 2, Table 9). In another experiment, formaldehyde-d_2 (Exp. 3) along with L-methionine-methyl-d_0 was administered to the organism. Two further experiments were performed in which non-labelled formate was fed at 1.5 x (Exp. 4) and at .75 x (Exp. 5) the concentration of methionine-methyl-d_3 to the organism. Finally, intracellular methionine was isolated from the aerobically grown cells (Exp. 1) and the bis-TMS derivative examined by GC/MS (Table 10).

From examination of Tables 9 and 10, it is evident that de novo methionine biosynthesis from formaldehyde-d_2


CD3
ATP
CD3
OH
NH2
S
O2
S
Ad
(CD3)2
N
CD2
OOH
OH
CD3
[H]
CD3-OH
CD2
OH
[O]
O2
CD2=O
H2N
N
N
HO
HN
O
C6H4
OH
NH
OH
O
D2C
HN
[H]
HD2C
HN
SH
CD2H
ATP
CDH
CD2
H
H
CD2H
S
Ad

Scheme 17

Table 9. Results of Experiments designed to examine the Role of One-Carbon Metabolism in D_1-Cyclopropane Fatty Acid Formation

Experiment #	Compounds Added to Basal Medium 2*	Cell Yield (mg/100 mL)	Deuterium Content of Biosynthetic Cyclopropane Fatty Acid[a]			
			% d_0	% d_1	% d_2	% $d_1/(\% d_1 + \% d_2)$
1 (aerobic)	methionine-methyl-d_3 (0.067 mM)	82	16.4	11.6	72.0	13.9
2 (anaerobic)	methionine-methyl-d_3 (0.067 mM)	120	15.7	12.2	72.0	14.5
3 (aerobic)	methionine (0.067 mM)	55				
	Formaldehyde-d_2 (0.60 mM)		100	0	0	0
4 (aerobic)	methionine-d_3 (0.34 mM)	178	16.9	12.7	70.4	15.3
	Formate (0.5 mM)					
5 (aerobic)	methionine-methyl-d_3 (0.67 mM)	135	16.8	15.3	67.9	18.4
	Formate (0.5 mM)					

* All culture media contained biotin at 10 µg/L.

Table 10

Results of the Methionine Isolation Experiment

Compound Analyzed	% d_0	% d_1	% d_2	% d_3
Methionine which was fed	14.0	0	1.0	85.0
Cyclopropane fatty acid isolated	16.4	11.6	72.0	
Methionine isolated	51*	1.0	0	48.0

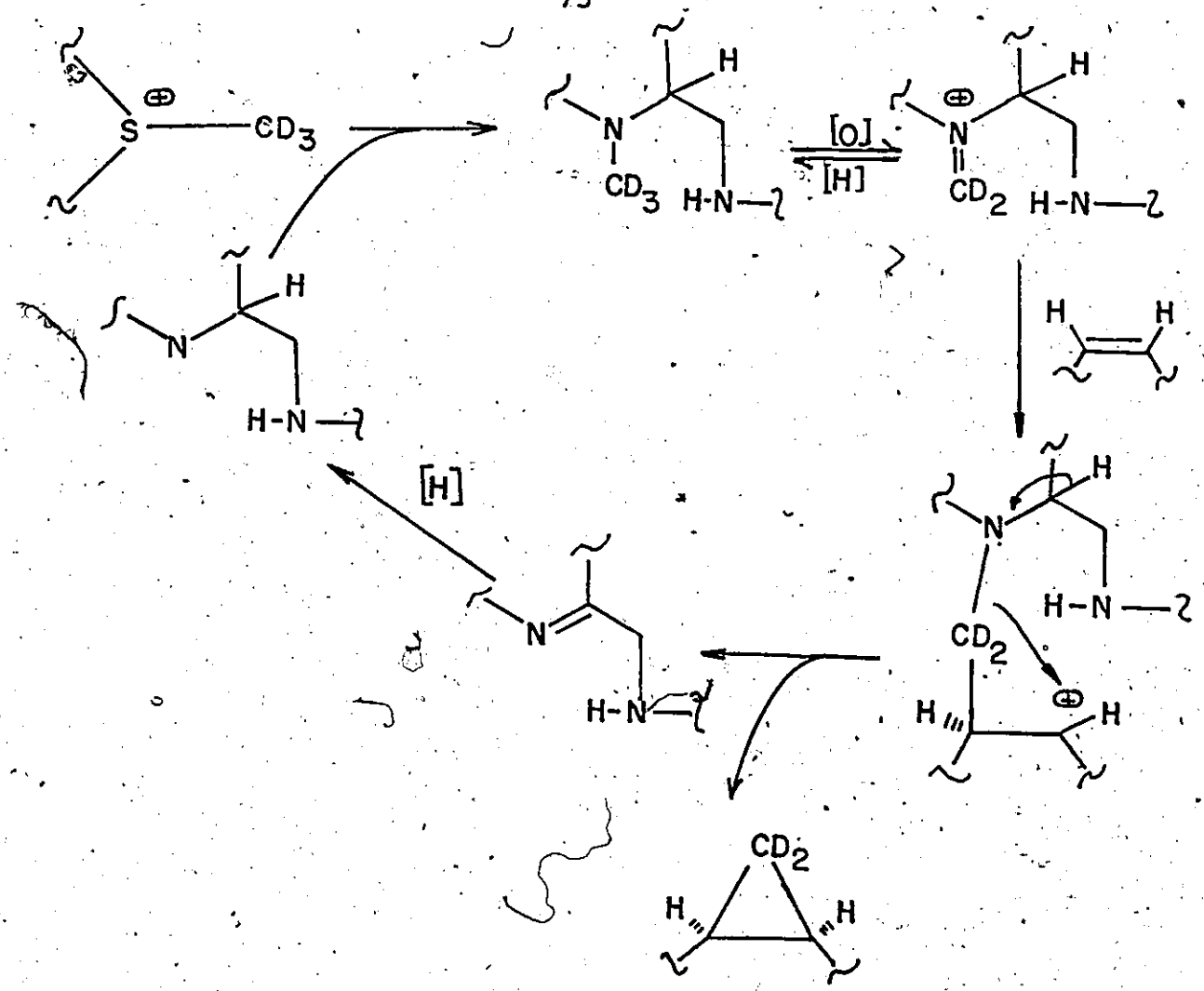
* The large amount of d_0-methionine was derived from the methionine tracer used to isolate the intracellular methionine.

is not involved in d_1-cyclopropane fatty acid formation.

Removal of oxygen from the culture medium did not reduce d_1-cyclopropyl fatty acid formation (Exp. 1, 2). Neither formate or formaldehyde was incorporated into the cyclopropane fatty acid (Exp. 3, 4, 5). Finally, reisolated methionine contained no d_1 or d_2-species (within experimental error, Table 10). These results are in accord with those of other workers who have found only low incorporation of "C_1" compounds into cyclopropane fatty acids (18).

(V) The preceding experiments do not rule out the intermediacy of tetrahydrofolate (T.H.F.) compounds, bound to an enzyme surface (thus preventing high incorporation of a one-carbon unit from the culture medium). One might envisage transfer of the methyl group from S.A.M. to enzyme-bound T.H.F., followed by an oxidation to generate 5,10-methylene T.H.F. An olefin attacked by the immonium ion might generate a carbonium ion which might collapse by loss of the C-6 proton of T.H.F. to form the cyclopropane ring. Reduction of 5,6-dehydro T.H.F. would complete the cycle (Scheme 18). Reversibility of 5,10-methylene T.H.F. formation would account for d_1-cyclopropane fatty acid production. However, a serious drawback to the above scheme is that there is no biochemical precedent for the methylation of T.H.F. by S.A.M.

(VI) It is possible that S.A.M.-methyl-d_3 loses deuterium

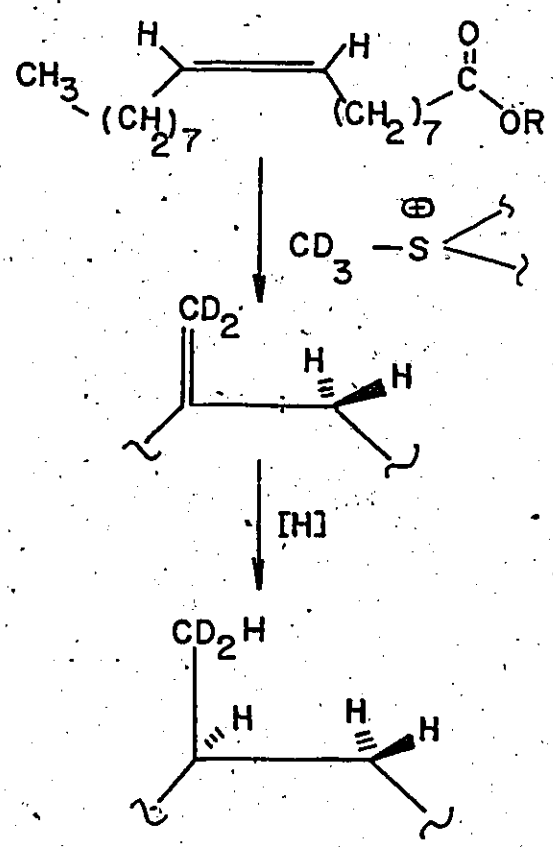


Scheme 18

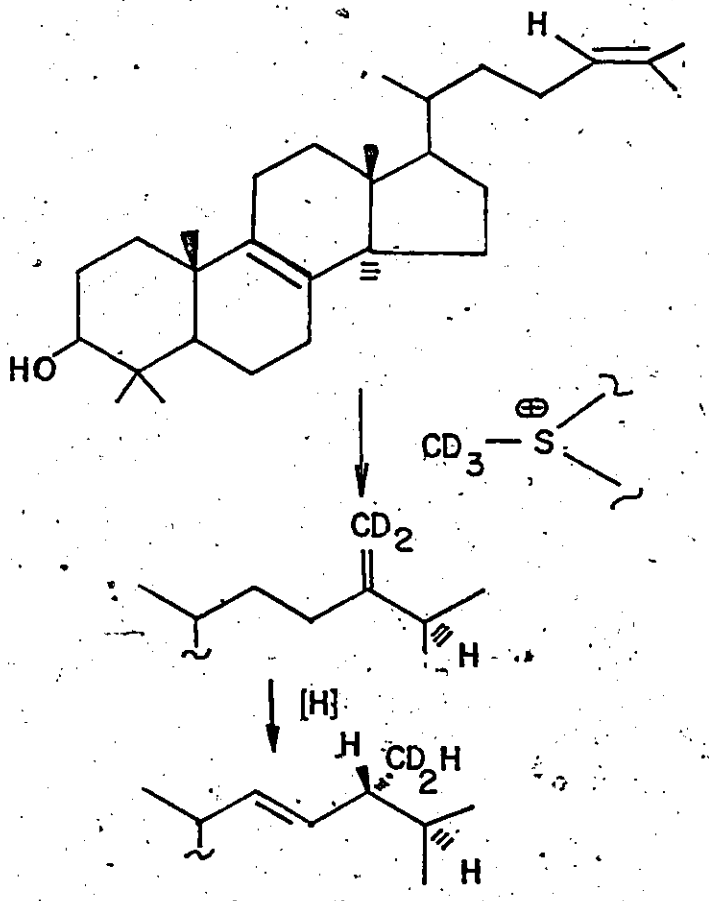
by a simple exchange process. This seems unlikely for a number of reasons. Firstly, the rate of exchange increases with cell growth, while the pH of the medium is constantly decreasing; these conditions are exactly opposite to those required for exchange of a methyl sulfonium salt. For example, NMR experiments have shown that S-methyl-methionine does not exchange its methyl protons in neutral medium. Secondly, S.A.M.-methyl-d_3 has been isolated from yeast (Fleischmann-

Standard Brands, Inc.) and shown to have the same deuterium content, within experimental error, as that of the methionine from which it was biosynthesized (60). Thirdly, natural products containing methyl groups derived from S.A.M.-methyl-d_3 via simple methyl transfer to electron-rich centres do not contain any significant d_2 or d_1-species (61-68).

(VII) The only known cases where exchange at the methyl groups has been documented are those involving the methylation of isolated double bonds such as in tuberculostearic acid or ergosterol biosynthesis (34, Scheme 19). Mercer et al. (69) have examined the deuterium content of intermediate 24-exo-methylene sterols derived from methionine-methyl-d_3 and have found that their d_1 content is very similar to that of ergosterol for which they serve as precursors (Scheme 20). The above authors attribute the presence of d_1-species to reversible hydrogenation of the exomethylene compounds and to methionine degradation and resynthesis. The former argument was first put forward by Lederer (34) who cited the production of euburicoic acid-d_2 (1) in Polyporus sulphureus and pachymic acid-d_2 (2) in Daedalea quercina from methionine-methyl-d_3 as examples of exomethylene formation occurring with very little exchange (% d_1/(%d_1 + %d_2) = 2%) (Figure 26). Lederer assumed that the above compounds were not metabolized further and concluded that the exchange process in ergosterol and tuberculostearate biosynthesis took place by reversible hydro-

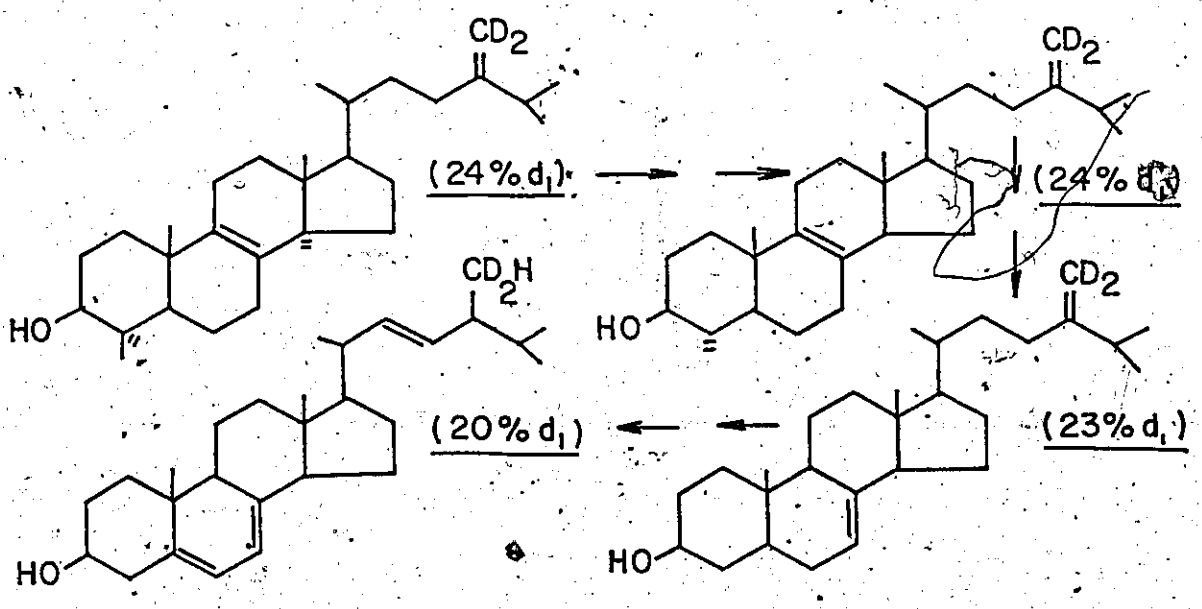


Tuberculostearic acid (11% d_1)



Ergosterol (16-19% d_1)

Scheme 19



Scheme 20

The numbers in parentheses represent % d_1/(% d_1 + % d_2) and are calculated from data in Refs. 34, 69, 75.

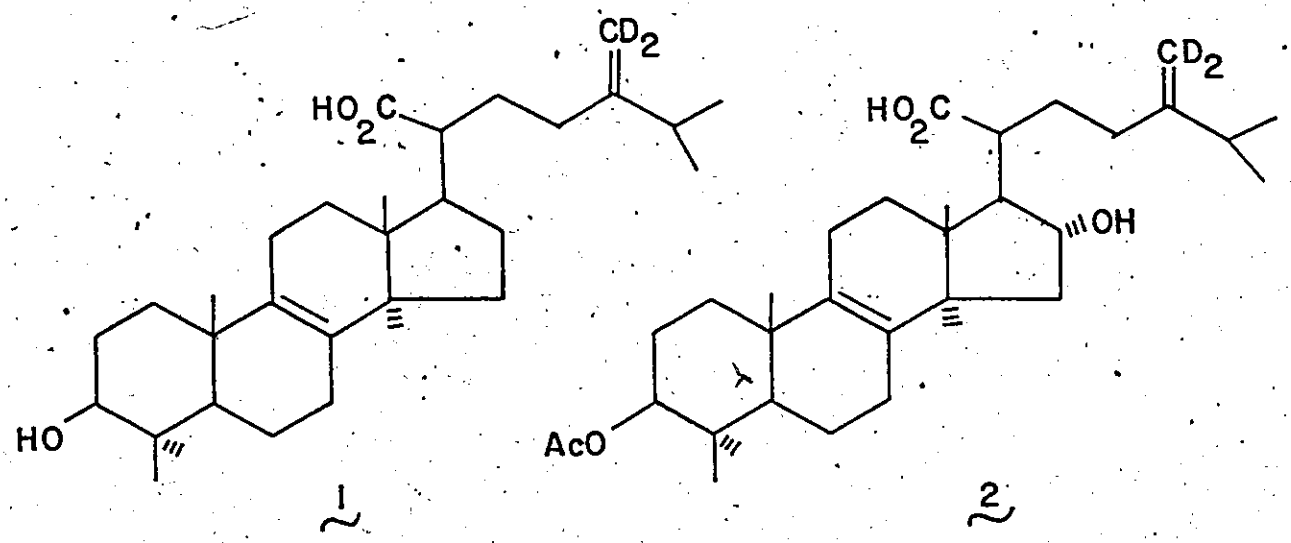


Figure 26. Euburicoic Acid and Pachymic Acid

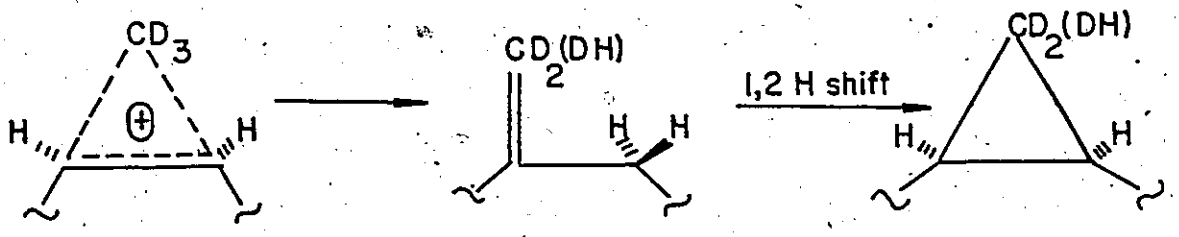
genation of the exomethylene intermediates. However, another interpretation is possible in light of the results presented in this thesis; namely, that exchange is occurring at the initial carbonium ion stage (see Scheme 1) by reversible cyclopropane formation, as originally suggested by P. De Mayo (34). The cases of euburicoic acid and pachymic acid biosynthesis cited above, simply demonstrate that the hydride shift leading to exomethylene formation can occur before reversible cyclopropane formation takes place.

Further support for the idea that exchange occurs at the initial carbonium ion stage, comes from an investigation of an interesting C-methylation of cis-vaccenic acid (or a derivative of this alkene), which leads to a 1:1 mixture of 7- and 8-methylheptadecane (70). A methionine-methyl-d_3 feeding was carried out with the blue-green alga, Anabaena variabilis, and it was stated that up to 20% of a d_2-species

accompanied the major d_3-methyl products (71). Although the detailed mechanism of this methylation reaction is not known, the presence of d_2-species could be explained by transient cyclopropane formation after methyl transfer. It should be noted that no olefinic intermediates have been found; a C_{17} cyclopropyl alkane has been detected in some algae species (72). Unfortunately, in other cases of "d_3-methylation" of an isolated double bond, it has not been possible to determine whether exchanged species are present (e.g., 73, 74).

One way of testing the hypothesis that exchange might be occurring via reversible cyclopropane ring formation would be to repeat the experiment of Altmann et al. (58, vide infra, p. 64) using a trideutero instead of a chiral methyl-oxidosqualene and looking for d_1-cycloartenol formation. Since no methyl transfer is involved in this system, any exchange that might occur, must have taken place by reversible cyclopropane ring formation.

(VIII) Another possible explanation of d_1-exomethylene group formation is that of Varenne et al. (75), who raised the possibility of reversible protonation of the exomethylene intermediate as a mechanism for exchange. The operation of this scheme in cyclopropane ring biosynthesis (Scheme 21) was tested for by feeding a mixture of 9- and 10-exomethylene-octadecanoic acid to L. plantarum. No appreciable cyclopropane ring formation from these compounds was observed.



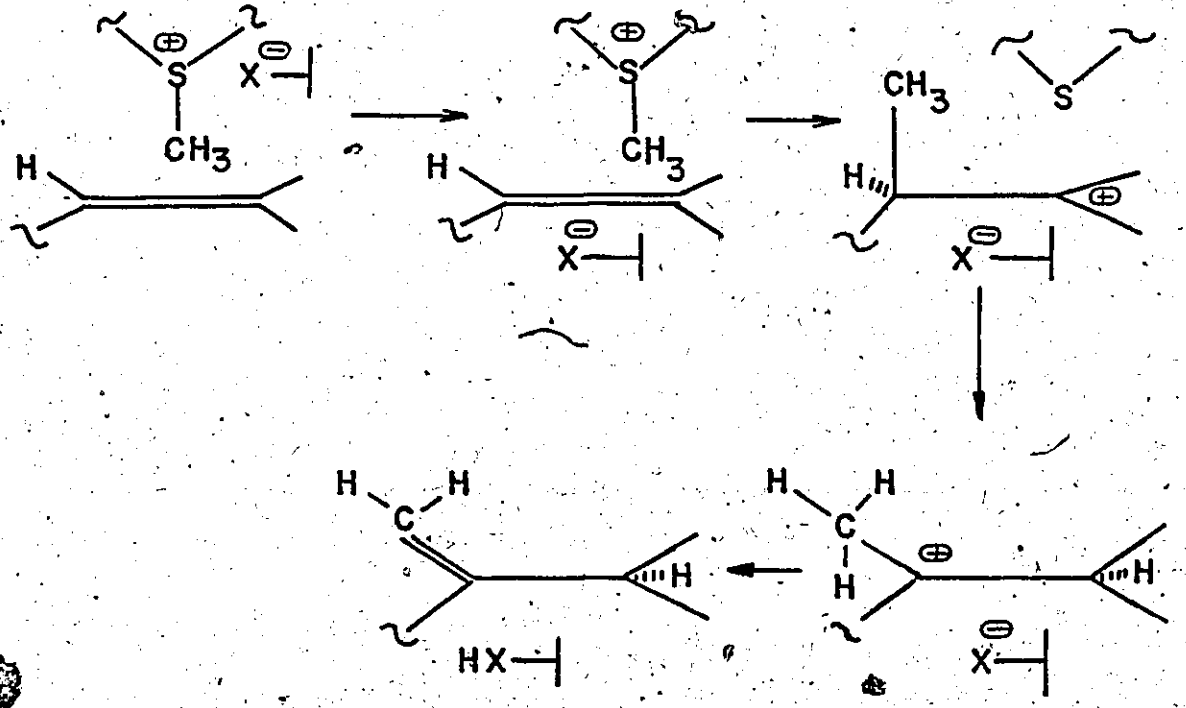
Scheme 21

(IX) From the above discussion, it is clear that this author favours reversible cyclopropane formation as the most probable mechanism for the exchange process in cyclopropane ring biosynthesis and indeed in all C-methylations of isolated double bonds. Yet, it is still possible to view the biosynthesis of cyclopropane fatty acids as occurring via a methylenation (carbenoid) mechanism rather than a methylation (carbonium ion) mechanism. Reversible sulfur ylid formation in the former case, prior to methylene transfer would account very nicely for the exchange process. It would be difficult to prove this experimentally since exchanged S.A.M. might not readily leave the enzyme active site. The only experiment which would give the carbenoid mechanism (and the above explanation for exchange) some credence would be to show that cyclopropane synthetase is, in fact, a metalloenzyme. This would require extensive purification of the enzyme; only very recently has any progress been made in this direction (24). One piece of evidence which suggests that cyclopropane synthetase is not a metalloenzyme, is the fact that in early work with crude en-

zyme preparations, cyanide ion was added to the extracts to prevent copper-catalyzed decomposition of S.A.M. (20); it might be expected that this would have inhibited cyclopropane synthetase if it were, in fact, a metalloenzyme (76).

I A MODEL FOR THE ACTIVE SITE OF CYCLOPROPANE SYNTHETASE

In conclusion, an active site model for the biological cyclopropanation reaction will be presented. As a starting point, the ideas of Akhtar and Jones (77), as further developed by Arigoni (78), will be used. The central idea is that electrophilic addition of a methyl group to an isolated double bond is initiated by an enzymic conformational change which separates the methyl sulfonium cation from its counterion (see Scheme 22). Because of the hydrophobic environment of

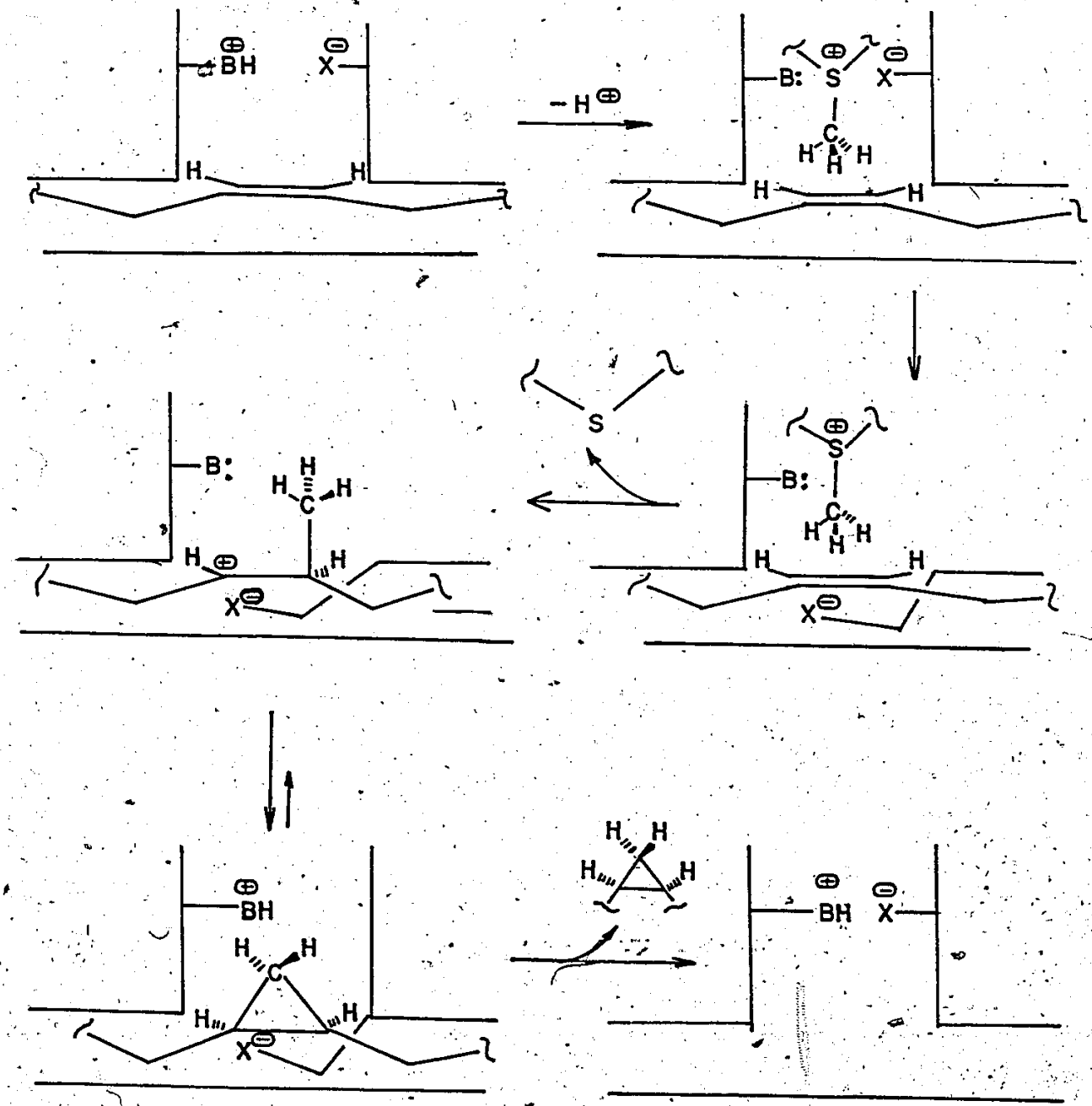


Scheme 22

the active site, this conformational change makes S.A.M. so potent a methyl donor that the weakly nucleophilic double bond is readily alkylated. The resultant carbonium ion can collapse in a variety of ways. To form exomethylene compounds (Scheme 22) a 1,2-hydride shift occurs followed by proton abstraction by the anionic counter ion to generate an overall neutral state. On the basis of stereochemical studies (78), Arigoni has postulated that the proton is removed from the plane of the olefin, opposite to that originally occupied by S.A.M. Thus, the initial conformational change must have brought the counter ion below the plane of the olefin.

The above arguments suggest that a hydrophobic pocket is a necessary condition for the methylation of isolated double bonds to occur. This idea receives some support from the fact that these reactions occur only on long chain fatty acids or sterol side chains; it is known that these olefins reside in the hydrophobic portion of the cell membrane. Also, the cyclopropanation reaction, when carried out <u>in vitro</u>, seems to occur only when the substrate lipids are in the correct micellar state (79).

The mechanistic picture outlined in Scheme 22 can be extended to explain why cyclopropane ring formation is reversible (Scheme 23). In this reaction, the base which removes the methyl proton must be on the same side of the double bond as S.A.M. This might be a neutral base (such as the ε amino group of lysine) which, when protonated, serves as the counter



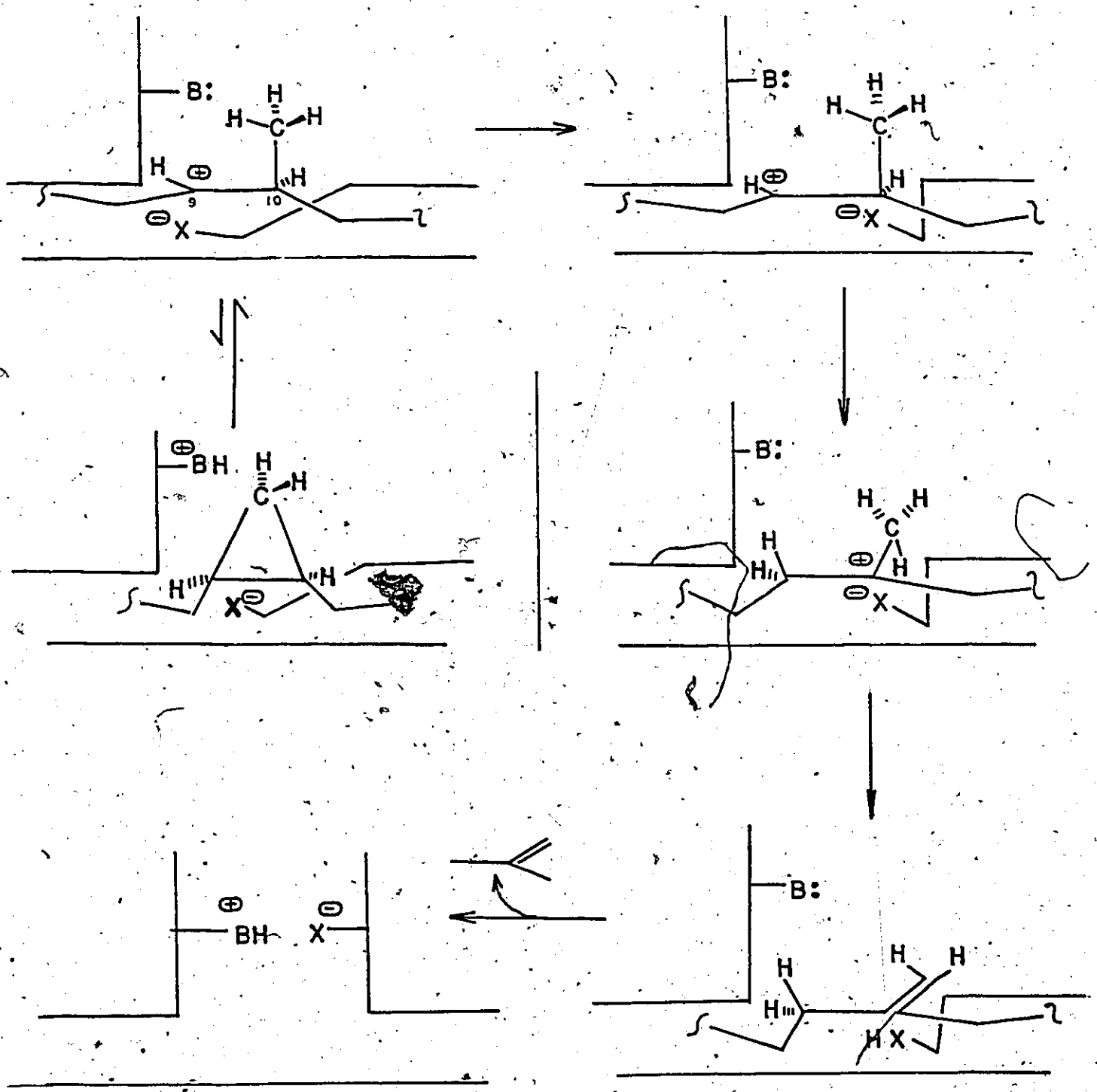
Scheme 23

ion for the anionic group in the absence of S.A.M. It is postulated that the anionic group is moved below the plane of the olefin to generate the charge-separated state as is thought to occur in ergosterol biosynthesis. In forming the cyclopropane ring, it can be seen from Scheme 23 that another charge-separated ion pair has been generated, thus presenting some driving force for the reverse reaction to occur as long as the product remains on the active site. The only other way of achieving "charge neutralization" is by forcing the product to leave the active site and so reform the "intimate ion pair". D_1-Cyclopropane product might result after only one opening and reclosure of the cyclopropane ring, since if the basic group has two or more equivalent hydrogens, then on reopening, a d_2-methyl group will result at least one time out of two. The cyclopropane ring cleavage reaction is an important biochemical reaction (80), as many phytosterols arise by ring opening the 9β,19-cyclopropane ring. If the ideas of Akhtar and Jones (77) on biochemical hydrogenation of an isolated bond are correct, then the cyclopropane ring cleavage reaction should occur by the mechanism shown in Scheme 23; i.e., an unsolvated BH^+ group should be a powerful enough acid to reopen the ring. The biochemical ring opening of obtusifoliol (a 9β,19-cyclopropyl sterol) has been carried out in D_2O, and since no deuterium was incorporated into re-isolated starting material, it was concluded that the cleavage reaction was not reversible (81). A better test for rever-

sibility would be to open obtusifoliol-19-d_2 in water and look for the presence of d_1-species in the product.

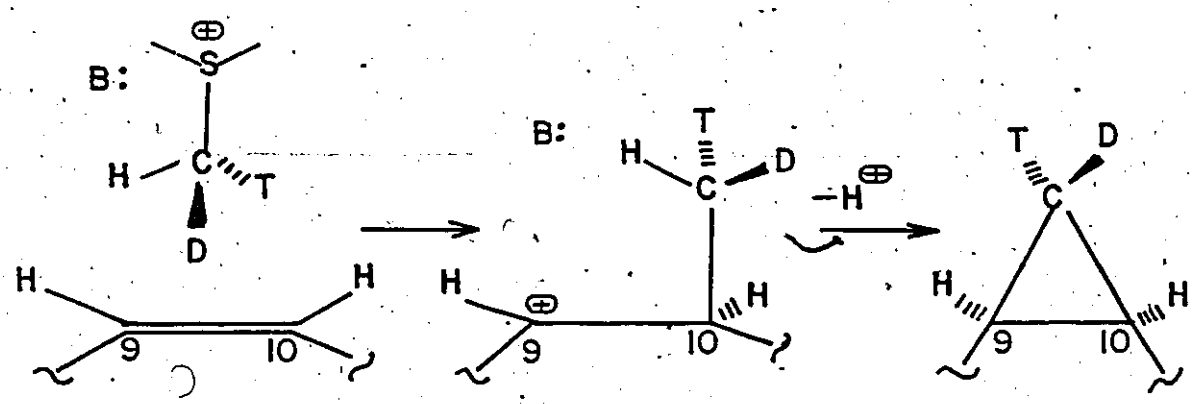
Using the ideas developed so far, it is easy to see why transient cyclopropane formation might occur in, for example, 10-exomethyleneoctadecanoic acid biosynthesis (Scheme 24). The essential difference between cyclopropane ring and exomethylene formation is the hydride shift from C-10 to C-9 in the latter case. This might be induced by movement of the anionic group from C-9 to C-10, thus encouraging development of positive charge at C-10. Since the methyl group cannot migrate, because of steric hindrance from the basic group, a hydride shift from C-10 to C-9 results. This has the effect of forcing the methyl group down near the anionic group, so as to allow proton abstraction to occur. The important point to be made is that before the hydride shift occurs, there is an opportunity for the carbonium ion at C-9 to collapse via cyclopropane ring formation, and so lead to exchange at the methyl group.

Based on the known similarities of the two enzymes responsible for dihydrosterculic and 10-exomethyleneoctadecanic acid formation (82), one might also expect very similar active site chirality in the two enzymes; i.e., C-9 and C-10 of an oleyl substrate would bear the same spatial relationship to the basic group (B). This leads to the conclusion that dihydrosterculate formation might occur via collapse of a carbonium ion at C-9. If this 1,3-elimination



Scheme 24

goes with retention of configuration as has been suggested (58), then one can make a prediction with respect to the outcome of a chiral-methyl-methionine feeding experiment. Thus, starting with L-methionine, containing a chiral methyl group of the "S" configuration, one would expect to obtain a biosynthetic dinydrosterculate having an "endo" deuterium and an "exo" tritium on the bridgehead carbon (Scheme 25).



Scheme 25

EXPERIMENTAL

A. General

Melting points were recorded on a Kofler micro hot stage apparatus and are uncorrected. Infrared spectra were obtained on a Perkin Elmer 238 spectrophotometer. Routine ^{1}H NMR spectra were recorded on a Varian E.M. 390 spectrometer. A Bruker WH-90 or WP-80 F.T. spectrometer was used for recording ^{1}H NMR spectra of small samples and obtaining all ^{2}H and ^{13}C NMR spectra. All chemical shifts are reported with reference to tetramethylsilane and are quoted in δ values. The symbols s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet are used in reporting spectra. Mass spectra were run either on a Consolidated Electrodynamics Corp. Model #21-110B mass spectrometer, at 70 ev using a direct introduction probe or on a VG Micromass 7070 instrument at 70 ev using a G.C. inlet system. The relevant instrumental parameters for the NMR work are listed in Table 11.

A variety of chromatographic techniques were employed in this work. Thin layer chromatography was performed using Merck precoated glass plates with silica gel 60 F-254 as stationary phase. Visualization of fatty acids was accomplished with the use of I_2 vapour or by spraying with a 1% solution of α-cyclodextrin in 30% ethanol, followed by exposure to I_2 vapour.

Table 11

Instrumental Parameters for F.T. N.M.R.

	^{1}H	^{2}H	^{13}C (normal)	^{13}C (microprobe)
Sweep Width (Khz)	1.2	1.0	6.0	6.0
Data Points (k)	4	4/8	8/16	4/8
Pulse Angle (°)	90	90	15-20	15-20
Pulse width (μs)	4	15-35	2.0-3.5	1.7
Acquisition Time (s)	4	4	0.5	0.5
Frequency (mHz)	90(80)	13.8	22.6	22.6
Sample Conc'n. (M)	0.02	0.02	0.3	0.3
Solvent Volume (μl)	1000	1000	1000	30
Ave. # of Scans	4	2000	30,000	100,000
Temp. (°C)	25	35	30	30

Gas-liquid chromatography of fatty acid methyl esters and TMS derivatives of amino acids was performed using a Pye Unicam Series 104 G.C. or a Varian Model 3700 G.C., both equipped with a flame ionization detector; a variety of packed columns coated with polar and non-polar stationary phases was employed. Capillary G.C. was performed on the Varian Model 3700 G.C., using a support-coated open tubular (SCOT) OV-101 column (100 m x 0.5 mm I.D.).

A Waters A.L.C. 100 liquid chromatograph, equipped with a refractive index detector, was used for high pressure liquid chromatography of fatty acids. A reverse phase column (Partisil O.D.S.-2, Magnum 9, 50 cm x 9.4 mm I.D.) was used for both preparative and analytical work.

Ion exchange chromatography was carried out, using a Beckman Model 119 automatic amino acid analyzer, equipped with a single column (32 cm x 0.9 cm I.D.), packed with Durrum D.C.-6A cation exchange resin.

The micro-organism used in this study was Lactobacillus plantarum, ATCC 8014, obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. The organism was grown at 37°C in a Precision Thelco hot air incubator and maintained as a stab culture at 4°C in a nutrient agar medium prepared by adding 2% agar to Lactobacilli M.R.S. broth (Difco). The biochemical and morphological characteristics of the organism were checked at the outset of this study and were found to be in accord with those described in the literature. Cells were

isolated by centrifugation either at 3500 r.p.m. on an International Clinical desk-top centrifuge for small scale experiments or at 5000 r.p.m. on a Beckman Model J-21 centrifuge equipped with a JA10 or JA20 centrifuge head. Cells were dried in a New Brunswick Sci. Co. Freezer dryer.

B. Synthetic Experiments

(I) Methyl cis-9-octadecenoate-9,10-d_2

9-Octadecynoic acid

Bromine (ca. 53 g) was added dropwise to an ethereal solution (400 mL) of oleic acid (100 g, Fisher 95% grade), at 0-5°C, until a yellow-orange colour persisted. A few drops of oleic acid were added to back titrate excess bromine. The dibromooctadecanoic acid, thus prepared, was stored until needed.

A 5 L, three-necked, r.b. flask was fitted with a gas inlet tube, a mechanical stirrer and a cold finger condenser charged with dry ice-isopropanol. The flask was cooled in dry ice and ammonia (1.9 L) was condensed into it over 3 h. Ferric chloride (1.6 g) was added to the vigorously stirred solution in one portion. Ten minutes later, sodium (3 g) was added, and the solution turned dark blue. Vigorous evolution of H_2 was observed and after it had ceased, the remainder of the sodium (40 g) was added in small pieces. Gray crystals of sodamide precipitated out of solution after 1 h. More ferric chloride (ca. 100 mg) was added until the solution

was no longer blue. The ethereal solution of the dibromooctadecanoic acid, prepared above, was added via a pressure-equalizing funnel in small portions and the reaction mixture stirred for a further 6 h, after which ammonium chloride (60 g) was added to destroy excess sodamide. The ammonia was allowed to evaporate overnight. Argon was continually passed over the mixture during the following operations. Water (1 L) was added to the solid residue and the dark brown mixture heated to 60°C to effect complete solution. Concentrated hydrochloric acid (50 mL) was added to render the solution acidic. The solution was cooled in an ice-bath before adding 500 mL of ether. The dark brown ether solution was separated, washed with water (2 x 600 mL) and dried over anhydrous sodium sulfate, the ether was then evaporated to give a dark brown oil which was dissolved in petroleum ether (500 mL). This mixture was filtered through Celite to remove a black precipitate, and the solution concentrated to 300 mL and stored at 5°C overnight. A tan coloured solid separated and was collected on a Buchner funnel. The filtrate was concentrated to 150 mL and cooled to yield a second crop. Yet a third crop was obtained by chilling the mother liquor. Recrystallization of the combined solids from petroleum ether (350 mL) yielded light tan crystals (38 g, 40% yield), m.p. 45-46°C, lit. m.p. 46-46.5°C (35).

ir (KBr disc): ν_{OH} - a br. band centred at 3000 cm^{-1}, $\nu_{C=O}$ 1700 cm^{-1}.

Raman: $\nu_{C\equiv C}$ 2226, 2299 cm^{-1}.

MS: m/e 280 (M^+).

^{1}H NMR ($CDCl_3$): 0.9 (3H,t,$\underline{CH_3}CH_2$), 1.3 (22H,m,$\underline{CH_2}$), 2.1 (4H, m, $\underline{CH_2}$-C≡C-$\underline{CH_2}$), 2.3 (2H,t,$\underline{CH_2}$COOH), 11.0 (1H, br.,COO$\underline{H}$).

^{13}C NMR: see Appendix II.

Methyl 9-octadecynoate

9-Octadecynoic acid (5.2 g) and a 14% BF_3/MeOH solution (100 mL) were heated under reflux for 0.5 h on a steam bath. When the solution had cooled, pet. ether (200 mL) and water (200 mL) were added and the mixture shaken in a separatory funnel. The pet. ether layer was separated, evaporated and the yellowish liquid residue (5.2 g) distilled at 130°C (0.1 mmHg) to yield methyl 9-octadecynoate (4.3 g, 83% yield) as a colourless liquid.

MS: m/e 294 (M^+), m/e 263 (M-MeO).

^{1}H NMR ($CDCl_3$): 3.65 (3H,s,COOC$\underline{H_3}$).

H.P.L.C. analysis (Acetonitrile/Ethyl Acetate (75:25), Flow rate = 1.5 ml/min) showed no trace of methyl oleate.

Catechol borane-B-d_1

An oven-dried, 250 mL r.b. flask was equipped with a stirring bar and a stopcock-controlled, septum-inlet adapter and allowed to cool in an ice bath under argon. A 1 M solution of BD_3 in tetrahydrofuran (49.5 mL) was introduced via syringe. Catechol (4.86 g) which had previously been dried in vacuo over P_2O_5, was dissolved in dry T.H.F. (10 mL) under argon and the solution transferred slowly via a transfer needle to the

reaction vessel with stirring. Stirring was continued at room temperature for 30 min, after which the reaction mixture was transferred via a needle to a vacuum distillation apparatus. The fraction boiling at 74°C (96 mm Hg) was collected. The colourless liquid (4.4 g, 81% yield) was transferred immediately via needle to a storage vessel equipped with a stopcock-controlled, septum inlet adapter and stored at -20°C over drierite. (An analogous preparation gave d_0-catechol borane with the following spectral data:)

^{11}B NMR (not referenced) ($CDCl_3$): 1 signal (d, J_{BH} = 195 Hz).

^1H NMR (neat): 7.3 (4H,m,arom.), 4.9 (1H,bd. q, J_{HB}=190 Hz, B-H), (latter signal observable at high R.F. only).

ir (film): ν_{B-H} 2685 cm^{-1}, ν_{O-B} 1480, 1240, ν_{C-O} 1135, ν_{C-H} 745 cm^{-1}.

MS: 120, 119 (M^+) due to ^{11}B, ^{10}B isotopes (MS contained extraneous peaks due to decomposition of the sample).

Methyl 9-cis-octadecenoate-9 or 10-d_1

All glassware and needles were heated in an oven overnight at 170°C. Methyl 9-octadecynoate (294 mg) was weighed into a 5 mL r.b. flask, which was then fitted with a stopcock-controlled septum inlet adapter. The reaction vessel was flushed with argon. Catecholborane (0.13 mL) was transferred via syringe to the reaction vessel. The mixture was stirred at 70°C and monitored via G.L.C. (3% OV-17 on 110-120 mesh Anakrom ABS, glass column, 4 mm I.D. x 7', T = 291°C, Flow

rate: 40 mL/min).

The reaction was essentially complete after 22 h. Acetic acid-d_4 (2 mL) was then added and the temperature increased to 100°C. In 9 h, the deuterolysis was judged to be complete by G.L.C. analysis. The reaction mixture was poured onto ice water and extracted with pet. ether and washed three times with 5% KOH, once with 5% HCl, dried over anhydrous sodium sulfate and the petroleum ether evaporated. The colourless residue was examined by ^{1}H NMR and mass spectrometry in order to assay its deuterium-content.

^{1}H NMR ($CDCl_3$): ratio of methyl ester protons (δ 3.65) to vinyl protons (δ 5.3) was 2.8:1; Product was 93% monodeuterated at the vinyl positions.

MS: By examination of the intensities of ions at m/e 297 (M^+:d_1) and m/e 296 (M^+:d_0), the following distribution of deuterium was calculated: d_1, 90%; d_0, 10%.

G.L.C. analysis of the product (15% OV-275 on Chromosorb P,T = 220°C, AW, DCMS (100/200), 20', x 1/8", S.S.) showed no trace of trans-9,10-olefin.

Methyl 9-cis-octadecenoate-9,10-d_2

The procedure used for the preparation of the title compound was identical with that described above except for the use of catechol borane-B-d_1 prepared from BD_3 (vide infra). Analysis by MS showed that the deuterated olefinic product had the following deuterium content: d_2, 67%; d_1, 30%, d_0, 3%.

Catalytic reduction of methyl 9-octadecynoate

Lindlar's catalyst was prepared in the following manner: calcium carbonate (1.25 g) was placed in a two-necked 50 mL r.b. flask, followed by D_2O (10 mL, 99.95% deuterated). The flask was attached to a hydrogenation apparatus via a reflux condenser. The apparatus was flushed with argon. Palladium dichloride (105 mg) was dissolved in D_2O (1.1 mL) containing DCl (0.1 mL) and the red solution added via syringe to the calcium carbonate suspension. The mixture was stirred for 5 min at room temperature and 10 min at 80°C, whereupon the apparatus was evacuated and filled with deuterium gas (D_2). The flushing procedure was repeated twice after which the solution was stirred and the absorption of D_2 at atmospheric pressure was monitored. When D_2 absorption had stopped (20 mL), the now grey suspension was filtered and resuspended in D_2O (12.5 mL) and a solution of lead diacetate (125 mg) in D_2O (2.5 mL) was added under argon. The mixture was stirred at room temperature for 10 min and at 100°C for 40 min. The catalyst was then filtered, washed with D_2O and dried over P_2O_5 in vacuo.

The reduction of methyl 9-octadecynoate was performed using deuterium gas at room temperature. The data obtained for the three experiments performed are summarized in Table 12 below. Cis/trans ratios were determined either by G.L.C. (see p. 95) or H.P.L.C. (see p. 90, mobile phase: acetonitrile (75)/ethylacetate (25), F.R. = 1.6 ml/min). The sample prepared in hexane was checked by ^{13}C, ^{1}H, and ^{2}H NMR

Table 12

Catalytic Reduction of Methyl cis-9-octadecynoate under 3 different conditions

Solvent (mL)	Scale (mmole)	% Trans	Time for Reaction (min)	Catalyst wgt (mg)	Deuterium Content $\%d_2$	$\%d_1$	$\%d_0$
Ethyl acetate (10)	1.0	8	38	25	93	6	1
Ethanol 0-D (5)	0.5	6	8	25	90	6	4
Hexane (5)	0.5	2	15	12	96	3	1

and the label was shown to be nearly 100% at the vinylic carbon atoms.

(II) Methyl cis-9-octadecenoate-8,8,11,11-d_4 Erythro-9,10-dihydroxyoctadecanoic acid

Oleic acid (95%, Fisher, 10 g) was dissolved in water (200 mL) containing potassium hydroxide (5 g). The mixture was heated on a steam bath and a further portion of potassium hydroxide (5 g) was added with stirring. The temperature was lowered to 5°C, by addition of crushed ice (175 g). Finely powdered potassium permanganate (10 g) was introduced and the reaction allowed to proceed with stirring for 10 min. Sulfurous acid was added until decolourization occurred. Acidification of the mixture with conc. hydrochloric acid gave a white precipitate that was collected, dissolved in hot ethanol (100 mL) and filtered.

The filtrate, on cooling, yielded crude diol (5 g) (m.p. 123-124°C). A further recrystallization from ethanol/benzene yielded pure erythro diol, m.p. 132°C, lit. m.p. 132° (44). ^{1}H NMR (DMSO-d_6): 0.85 (3H,t,CH_3-CH_2), 1.25 (26H,m,CH_2), 2.25 (2H,t,CH_2COO), 3.15 (2H,m,CHOH), 3.5 (2H,OH). ^{13}C NMR: (see Appendix I).

Attempted deuterium exchange of erythro-9,10-dihydroxyoctadecanoic acid

Potassium metal (2.78 g) was dissolved in tert-butyl alcohol-O-d (25.5 g). The alkoxide solution was added to the

dihydroxy acid (158 mg) along with fluorenone (5 mg) and fluorenol (10 mg). The mixture was heated in a sealed tube at 180°C for 24 h. The reddish-brown reaction mixture was acidified with 5% HCl, excess alcohol evaporated and the diol extracted with ether. The dihydroxy acid was isolated by prep. T.L.C. (10% MeOH/$CHCl_3$) and a ^{13}C NMR of the sample was run. No reduction in intensity of the signal assigned to the carbon atoms alpha to the hydroxy functions was observed. (The signal assigned to the carbon atom alpha to the carboxyl group had disappeared.)

9,10-Dioxooctadecanoic acid

Oleic acid (88 g, 95%, Fisher) was dissolved in acetic anhydride (1 L) in a 5 L, 3-necked, r.b. flask, fitted with a mechanical stirrer. The mixture was cooled in an ice-salt bath to -5°C. Pulverized potassium permanganate (251 g) was added over a period of 1 h with stirring, keeping the temperature below 5°C. Stirring was continued for another 90 min. a 2:1 (v/v) ethyl acetate/hexane mixture (1.5 L), previously cooled to -10°C, was added to the reaction mixture. Sodium bisulfite (100 g) was dissolved in water (0.5 L), mixed with saturated sodium chloride solution (0.5 L) and chilled to -5°C, before adding to the reaction vessel. The resulting dark liquid was poured into a large separatory funnel and the aqueous layer drawn off. The organic layer was washed once more with cold bisulfite solution to render it yellow and washed several more

times with water. The solvents were removed in a rotary evaporator at 60°C along with excess acetic anhydride. The yellow residual oil was dissolved in pyridine (ca. 50 mL) and cooled in an ice bath. Water was carefully added to hydrolyze the fatty acid anhydride. The solution was stirred overnight at room temperature to complete the hydrolysis. Ether (2 L) was added and the pyridine removed by washing several times with dilute hydrochloric acid. The ether was removed in vacuo and the residue recrystallized from 95% ethanol (1 L) to give 9,10-dioxooctadecanoic acid as yellow plates (30 g, 34% yield), m.p. 81-82°C, lit. m.p. 85°C (46).

^{1}H NMR ($CDCl_3$): 9 (3H,t,CH_3CH_2), 1.2 (26H,m,CH_2), 2.35 (2H, t,CH_2COOH), 2.7 (4H,t,CH_2COCO), 11.0 (1H, COOH).

ir ($CDCl_3$): ν_{OH} 3000 cm^{-1}, ν_{CH} 2915, 2060 cm^{-1}, $\nu_{C=0}$ 1710 cm^{-1}.

MS: m/e 312 (M^+) m/e 169 ($M-CH_3-(CH_2)_7-CO$), m/e 141 (further loss of CO).

9,10-Dioxooctadecanoic acid-8,8,11,11-d_4

Acetic anhydride, distilled through glass helices (102 g), was placed in a three-necked, 500 ml, r.b. flask fitted with a reflux condenser and an addition funnel. Deuterium oxide (99.7% D, 40.0 g) was added and the reaction mixture stirred and heated until an exothermic reaction set in at which point heating was halted. When the hydrolysis was complete, the flask was cooled in ice and acetyl chloride (19.6 g) was added

slowly. The final mixture contained acetic acid-O-d (2.25 moles), D_2O (0.75 moles), DCl (0.25 mole). 9,10-Dioxooctadecanoic acid (7.8 g) was added and the mixture heated to 65°C for 0.5 h. The solvent was removed *in vacuo* and the exchange repeated once more with fresh medium. After workup, ^1H NMR showed that the intensity of the signal at 2.7 ppm had been greatly reduced. The sample (7.8 g) was dried *in vacuo* to remove traces of DCl and D_2O, and used directly in the next step.

9,10-Dihydroxyoctadecanoic acid

Platinum oxide (500 mg), suspended in acetic acid (10 mL) in a 250 mL r.b. flask, was prehydrogenated, after which the tetradeutero diketone (7.8 g) prepared above was added in acetic acid (50 mL) and the hydrogenation allowed to proceed over 22 h. 1,090 mL of Hydrogen was absorbed (97% of theoretical). The white solid which had precipitated out of solution was dissolved by adding acetone and the solution was filtered from the catalyst. The solvents were removed *in vacuo* and an aliquot of the residue (m.p. 115°C) was methylated with diazomethane and analyzed via boric acid impregnated silica gel T.L.C. (mobile phase 60/40 ether/pet. ether; the T.L.C. plates were prepared by spraying Merck silica gel plates with a saturated methanolic boric acid solution). This analysis showed that the major product was the erythro diol by comparison with an authentic sample. Two recrystallizations from ethyl acetate yielded pure erythro diol, 5 g, m.p. 126-128°C, lit. m.p. 132°C (44).

Methyl cis-9-octadecenoate-8,8,11,11-d_4

Erythro 9,10-dihydroxyoctadecanoic acid-8,8,11,11-d_4 (5 g) was heated with methyl orthoformate (6 mL) under argon at 90°C for 0.5 h. Excess methyl orthoformate was evaporated and the resulting 1,3-dioxalan heated at 160°C for 4.75 h, at which time, the evolution of carbon dioxide had ceased. The product was shown by T.L.C. to be mainly oleic acid, accompanied by some methyl oleate. The product was treated with diazomethane to convert the acid to its methyl ester which was isolated by dissolving the crude product in chilled hexane and centrifuging to remove a large amount of gelatinous precipitate. Removal of the solvent gave a residue of 3.9 g. Open column chromatography using Merck T.L.C. silica gel (100 g) and elution with ether/hexane (2:23) yielded the pure title compound. G.L.C. analysis (c.f. p. 95) showed the product to be the stereochemically pure cis-olefin. Analysis of the ^1H, ^2H and ^{13}C NMR spectra showed that the label was exclusively at the allylic positions. Analysis of the product by MS showed that it had the following deuterium content: d_4, 79.1%; d_3, 15.1%; d_2, 4.0%; d_1, 1.4%; d_0, 0.4%.

Reduction of 9,10-dioxooctadecanoic acid with Sodium Borohydride

9,10-Dioxooctadecanoic acid (5 g) was dissolved in methanol (300 mL) and treated with sodium borohydride (5 g) in small amounts over 10 min. The colourless solution was stirred at room temperature for an additional 3 h. Water

(200 mL) was added and the solution acidified with acetic acid to yield a white precipitate (5 g), which was filtered off. The white solid was extracted in a Soxhlet extractor with ether. Both the ether soluble and the ether insoluble fraction were shown by boric acid-T.L.C., to contain an excess of the threo diol. Both fractions after recrystallization from ethyl acetate melted at 93°C. M.p. of the pure threo diol is 93°C (47).

(III) Miscellaneous Preparations

Methyl cis-9,10-epoxyoctadecanooate

Methyl cis-9-octadecenoate (10 g), was dissolved in methylene chloride (100 mL) and the solution cooled in an ice bath at 0°C. m-Chloroperbenzoic acid (90%, 7 g) in methylene chloride (100 mL) was added dropwise with stirring. After 0.75 h, a white precipitate had formed and the reaction was judged to be complete by ^{1}H NMR examination of an aliquot of the reaction mixture. The methylene chloride solution was warmed and washed with 5% sodium bicarbonate and 10% sodium sulfate. Evaporation of the solvent yielded a colourless oil (10.3 g, 98% yield).

^{1}H NMR ($CDCl_3$): 2.9 (2H,m,C-9H,C-10H).

MS: m/e 312 (M^+), m/e 280 ($M-CH_3OH$), m/e 199 ($M-C_8H_{17}$), m/e 155 ($M-(CH_2)_7COOCH_3$).

Methyl 9-methyl-10-hydroxyoctadecanoate and methyl 10-methyl-9-hydroxyoctadecanoate

Cuprous iodide (1.07 g) was dissolved in dimethylsulfide (4 mL) and the resulting pale yellow solution was filtered through glass wool from a black precipitate, into a three-necked 100 mL, r.b. flask, equipped with two rubber septa and a gas inlet adapter leading to a mercury bubbler and a source of argon. Dimethylsulfide (10 mL) and ether (15 mL) were added and the flask cooled in an ice bath before flushing with argon. Methyl lithium (1.7 M in ether, 6.3 mL) was added via syringe until the initial bright yellow precipitate had dissolved. Methyl cis-9,10-epoxyoctadecanoate (835 mg) in ether (10 mL) was added to the reaction flask. The reaction mixture was stirred at 0°C for 13 h. An ammonium hydroxide/ammonium chloride buffer solution, pH 8) was added and the blue solution extracted with ether. The excess dimethyl sulfide was removed in vacuo and the organic layer was washed with buffer until colourless. The ether was removed in vacuo and the residue chromatographed using reverse phase H.P.L.C. (cf. p. 90). The two major components were isolated in the following manner: elution of the crude mixture with acetonitrile/water (90:10) yielded the faster moving component (compound #1) in pure form (120 mg). Rechromatographing the second component using methanol/water (95:5) as mobile phase was necessary to obtain Compound #2 in a pure state (120 mg). Total yield: 27%.

Both compounds had identical ^{1}H and ^{13}C NMR and ir spectra;

^{1}H NMR ($CDCl_3$): 3.5 (1H,m,C<u>H</u>OH), 0.8 (6H,m,C<u>H</u>$_3$CH$_2$ and C<u>H</u>$_3$-CH-)

^{13}C NMR: (see Appendix I)

ir ($CDCl_3$): ν_{OH} 3650 cm^{-1}, $\nu_{C=O}$ 1745 cm^{-1}.

The compounds were identified on the basis of the fragmentation pattern in their mass spectra.

MS: Compound #1: m/e 328 (M^+), m/e 297 ($M-CH_3O$), m/e 215 ($M-C_8H_{17}$), m/e 186 ($M-CH_3(CH_2)_7COH$, Base pk), m/e 143 ($M-HC(CH_3)-(CH_2)_7-CO_2CH_3$)

Compound #2: m/e 328 (M^+), m/e 297 ($M-CH_3O$), m/e 187 ($M-CH_3-(CH_2)_7-C(H)CH_3$), m/e 155 (loss of MeOH from ion at m/e 187, base pk).

Based on the above evidence, compound #1 was assigned the structure, methyl 9-methyl-10-hydroxyoctadecanoate and compound #2 the structure, methyl 10-methyl-9-hydroxyoctadecanoate. On the basis of the method of preparation, it was assumed that both compounds had the threo configuration at C-9 and C-10.

Hydrolysis of the ester function of each compound (25 mg) was accomplished by heating them under reflux in a 50% ethanol-water (2 mL) solution containing potassium hydroxide (200 mg) for 0.5 h. The ethanol was removed <u>in vacuo</u> and water (5 mL) added. The acids were extracted with ether, dried over sodium sulfate, and the ether removed <u>in vacuo</u> to yield the acids (20 mg). The fatty acids were checked by T.L.C. and ^{1}H NMR

and found to be free of traces of the ester.

Methyl 9-oxooctadecanoate and Methyl 10-oxooctadecanoate

Methyl cis-9,10-epoxyoctadecanoate (2.0 g) and boron trifluoride dietherate (2.82 gm) were dissolved in 1,4-dioxan (100 mL) and the mixture refluxed at 115°C for 3 h. The solution was cooled and poured into benzene (80 mL) and washed 10 times with water (300 mL). The benzene layer was dried over anhydrous sodium sulfate before removing the benzene in vacuo. The residual yellowish oil (1.9 g) crystallized on standing (67% yield). Reverse phase H.P.L.C. using acetonitrile/ethyl acetate (75/25) showed essentially an equimolar 2 component mixture which was only slightly resolved on a Magnum 9, Partisil ODS-2 column.

^{1}H NMR ($CDCl_3$): 2.4 (4H,t,$(CH_2)_2C=O$).

Methyl 9-exomethyleneoctadecanoate and Methyl 10-exomethyleneoctadecanoate

Sodium hydride (0.5 g, 50% oil dispersion) was weighed into a three-necked, 100 mL, r.b. flask, fitted with two rubber septa, a stirring bar and a reflux condenser equipped with a 3-way stopcock-controlled gas inlet adapter. The sodium hydride was washed several times with n-pentane and the system evacuated and refilled with argon several times. Dimethylsulfoxide (dried over sodium hydride, 5 ml) was added via syringe. The mixture was stirred and heated at 80°C until hydrogen evolution ceased. (A yellowish-green precipitate had formed.)

Methyl triphenyl phosphonium bromide (3.6 g) was dissolved in dry dimethyl sulfoxide (10 mL) under argon and the solution transferred via syringe to the cooled dimsyl anion solution. The solution turned red immediately. After 0.5 h, 6 mL of the above solution was added to a 1:1 mixture of methyl 9-oxo-octadecanoate and methyl 10-oxooctadecanoate in dimethylsulfoxide (2 mL) under argon. The solution was warmed slightly to ensure dissolution of the esters and the mixture stirred for 20 h. The still reddish solution was quenched with water (100 mL) and extracted with pentane (3 x 100 mL). The pentane layer was washed with water/dimethylsulfoxide (1:1, 30 mL), then with water (100 mL), and finally dried over anhydrous sodium sulfate. The residue obtained upon evaporation was examined by reverse phase H.P.L.C. (acetonitrile/EtOAC 75:25) and shown to be free from traces of starting material. A small aliquot of the mixture was isolated by H.P.L.C. (same mobile phase as above) to remove traces of triphenylphosphine oxide. Separation of the two regioisomeric exomethylene compounds was not possible using the above H.P.L.C. conditions.

^{1}H NMR ($CDCl_3$): 2.0 (4H,m,$C\underline{H}_2$-C=CH_2), 4.6 (2H,brd s,$C\underline{H}_2$=C).

^{13}C NMR ($CDCl_3$): (see Appendix I).

ir (CCl_4): $\nu_{C=O}$ 1750 cm^{-1}, $\nu_{C=C}$ 1650 cm^{-1}.

Preparation of methyl cis-9,10-methyleneoctadecanoate

Zinc dust (400 mg, Analar B.D.H.) was suspended in boiling acetic acid in a 10 mL r.b. flask. The mixture was stirred for 1 min, cupric diacetate (80 mg) in boiling acetic

acid (2 mL) was added and stirring continued for ca. 3 min until the blue solution had been completely decolourized. The red-brown zinc-copper couple was washed with acetic acid (5 x 3 mL) and ether (5 x 3 mL). Ether (1 mL) was then added, followed by diiodomethane (2.5 g), and methyl cis-9-octadecenoate (100 mg) in ether (4 mL). A reflux condenser was fitted to the flask and the system flushed with argon. The ether solution was warmed to initiate the reaction and after an initial exothermic reaction had subsided, the solution was heated under reflux for 24 h, under argon, at an oil bath temperature of 55°C. (After 12 h, ether (2 mL) was added to replace that lost by evaporation.) Pyridine and ether were added to the cooled reaction mixture until a white precipitate ceased to form. The mixture was filtered and the solids washed with ether. The yellow residue, obtained by evaporation of the ether, was placed under high vacuum (0.01 mm Hg) and excess pyridine and diiodomethane removed. The residual oil was taken up in hexane and filtered through glass wool to remove an orange solid. Reverse phase H.P.L.C. analysis (Acetonitrile/ethyl acetate 75/25) showed essentially one peak assigned to the cyclopropane compound.

^{1}H NMR: see p. 36.

^{13}C NMR: see p. 39.

MS: m/e 310 (M^+), m/e 278 ($m-CH_3OH$)

ir (CCl_4): ν_{C-C} 1015 cm^{-1} due to the cyclopropyl group.

Preparation of L-methionine-methyl-d_1

S-Benzyl-L-homocysteine (0.5 g) was weighed into a 100 mL three-necked, r.b. flask which was then fitted with a cold finger condenser, a gas inlet tube and a rubber septum. Both the condenser and the gas inlet tube were guarded by in-line potassium hydroxide drying tubes. The flask was cooled in a dry ice-acetonitrile bath and the condenser was charged with a dry ice-acetonitrile slurry. Ammonia (15 mL) was condensed into the flask and the solution stirred to dissolve the S-benzyl-L-homocysteine. Small pieces of sodium were added to the mixture until the solution remained dark blue for a few minutes. At this point, the mixture had a turbid, white appearance.

Methyl iodide-d_1 (150 µL) was added via syringe, the mixture stirred for 10 min, and the ammonia allowed to evaporate by removing the condenser. Water (5 mL) was added to dissolve the white solid and the pH of the solution was adjusted with hydriodic acid (47%) until it was acidic to litmus but alkaline to Congo red. Toluene and a few ml of water were removed *in vacuo*. Boiling ethanol (30 mL) was added to the residue and the solution allowed to stand overnight at 5°C. The silvery-white solid, which separated, was filtered, washed with cold ethanol and ether, and dried to constant weight (259 mg, 80% yield) *in vacuo* over P_2O_5.

^{1}H NMR (D_2O): 2.1 (4H,m,$C\underline{H}_2CH(NH_2)CO_2H$ + $C\underline{H}_2D$-S), 2.6 (2H,m, CH_2-S), 3.8 (1H,t,$NH_2C\underline{H}$-COOH).

From analysis of the bis TMS derivative of the title compound at m/e 293 (M^+,d_0) and m/e 294 (M^+,d_1), the following deuterium content was calculated: d_1, 97%; d_0, 3%.

Preparation of L-methionine-methyl-d_2

The title compound was prepared by a method identical with that described above, except for the use of methyl iodide-d_2.

^{1}H NMR (D_2O): 2.1 (3H,m,$C\underline{H}_2$-CH(NH_2)COOH,$C\underline{H}D_2$-S).

MS: By a similar analysis to that described above, the following deuterium content was calculated: d_2, 96.7%; d_1, 2.7%; d_0, 1.6%.

Preparation of a 0.1% Dideutero-formaldehyde solution

Paraformaldehyde-d_2 (a generous gift of Prof. D. Moule) (3.75 mg) was suspended in water (4 mL) and heated under reflux for 15 min, at which time the white solid had disappeared. The reaction mixture was washed into a 10 mL Erlenmeyer flask, dimedone (10% w/v in 95% ethanol, 0.35 mL) added and the solution allowed to stand overnight. The white precipitate was collected in a Buchner funnel and weighed after drying *in vacuo*. Theoretical yield: 38.7 mg; Actual yield: 35.4 mg. The product showed one uv active spot on silica gel T.L.C. (100% chloroform) corresponding to an authentic sample of the bis dimedone derivative of formaldehyde.

^{1}H NMR ($CDCl_3$): 1.1 (12,s,C-($C\underline{H}_3$)$_2$), 2.3 (8,s,$C\underline{H}_2$-C=O).

The signal at δ 3.2 assigned to $C\underline{H}_2$-C=C(OH) in the non-deuterated compound was missing as expected.

C. Biosynthetic Experiments

(I) Media

The basal media used in the feeding experiments were either Medium A or Medium B; their compositions are listed below.

Medium A (1 L)

Constituent	Amount
Bacto-Biotin Assay Medium (Difco)	37.5 g
10% Vitamin-Free Casein Hydrolyzate solution	50.0 mL

Medium B (1 L)

Constituent	Amount
Glucose	20.0 g
Sodium citrate	20.0 g
Sodium acetate	1.0 g
Ammonium chloride	3.0 g
Potassium hydrogen phosphate	5.0 g
Salts solution A	20.0 mL
Base solution B	10.0 mL
Base solution C	10.0 mL
Vitamin solution D	10.0 mL
L-alanine	0.5 g
DL-aspartic acid	1.0 g
L-arginine hydrochloride	0.2 g
L-lysine hydrochloride monohydrate	0.2 g
L-histidine	0.1 g
L-isoleucine	0.1 g

Constituent	Amount
L-leucine	0.1 g
DL-phenylalanine	0.2 g
L-proline	0.1 g
DL-threonine	0.2 g
L-valine	0.1 g
DL-tryptophan	0.2 g
DL-serine	0.1 g
L-tyrosine	0.1 g
L-cystine	0.1 g
Glycine	0.1 g

Salts solution A (250 mL)

Constituent	Amount
Magnesium sulfate heptahydrate	10.0 g
Ferrous sulfate heptahydrate	0.5 g
Sodium chloride	0.5 g
Manganese sulfate heptahydrate	2.0 g

Base solution B (100 mL)

Constituent	Amount
Adenine sulfate	0.1 g
Guanine hydrochloride	0.1 g
Uracil	0.1 g

Base solution C (100 mL)

Constituent	Amount
Xanthine	0.1 g

Vitamin solution D (1 L)	Amount
Thiamine	100.0 mg
Riboflavin	100.0 mg
Pyridoxal	20.0 mg
Calcium pantothenate	100.0 mg
Niacin	100.0 mg
p-Aminobenzoic acid	20.0 mg
Folic acid	1.0 mg

2M HCl

L-Tyrosine and L-cystine were first dissolved in the minimum amount of 2M HCl. The resulting solution was diluted and the other components were then added. Base solution B was made up with the aid of 1 M HCl. Base solution C was made up in 1 M NH_4OH. The final pH of the medium was adjusted to 6.8 with 1M KOH.

(II) Extraction of Microbial Cells and Isolation of Fatty Acids

Procedure (a): Large Scale Experiments (16)

Microbial cells (0.5 - 1.0 g, dry wgt.) were placed in a 100 mL r.b. flask and heated under reflux, under argon, for 5 h, using a hydrolysis mixture containing potassium hydroxide (2.25 g) dissolved in 63% ethanol (47.5 mL). The ethanol was removed *in vacuo*, after the hydrolysis was complete, and the basic residue washed once with ether (25 mL) and then acidified to Congo red with 50% sulfuric acid. The fatty acids were extracted with ether (4 x 20 mL) and the ether layer was washed with water (2 x 20 mL) and then dried over anh. sodium sulfate. Upon evaporation of the ether, the fatty acids were methylated with a freshly prepared ethereal solution of diazomethane. The methyl esters were dried over

P_2O_5 *in vacuo* before chromatography on a reverse phase H.P.L.C. column was performed (Acetonitrile/Ethyl acetate (75:25), Flow rate, 1.6 mL/min). The retention times of the three major fatty acids in the extract were as follows: methyl cis-vaccenate (43 min), methyl palmitate (46 min) and methyl lactobacillate (50 min). Base line resolution was obtained for this mixture, when the above conditions were employed.

Procedure (b): Extraction of cells for growth curve studies (27)

Aliquots of the microbial culture (10 mL) were centrifuged, and the cell pellet was stored at -60°C. The cells were brought to 50°C and a chloroform/methanol (2:1) mixture was added to the cell pellet. The mixture was sonicated for 30 sec and allowed to stand for 15 min. A 0.1 M sodium chloride in 0.1 M hydrochloric acid solution (1 mL) was added and the biphasic mixture was agitated using a desk-top test tube mixer. The two phases were separated by centrifugation at 3500 r.p.m. for 10 min. The lower layer was carefully removed with a pipette and transferred to a 25 mL r.b. flask and the chloroform evaporated at 35°C. Sodium (300 mg) was dissolved in methanol (25 mL) and an aliquot (2 mL) of the resulting 0.5 M solution was added to the extract. The methanolysis was allowed to proceed for 15 min at room temperature. Hexane (4 mL) was then added, with shaking, followed by 3 M sulfuric acid (0.2 mL) and water (4 mL). The two phases were poured into a 15 mL centrifuge tube and the top layer removed via

pipette and passed through sodium bicarbonate/sodium sulfate (1:2) into another 15 mL centrifuge tube. The hexane was evaporated under argon and the residue taken up in n-pentane (10 µl). An aliquot (1 µl) was injected onto a G.C. column (7' x 2 mm I.D., glass, 12% DEGS on Gas chrom Q (80/110), isothermal at 170°C). Peak areas were measured by triangulation.

(III) Feeding Experiments

In most of the feeding experiments to be described, a freshly grown inoculum was used. One or more culture tubes containing M.R.S. Broth (Difco, 10 mL) was inoculated from a stock stab culture. After 24 h of growth, the cells were harvested, washed with 0.85% saline several times, and resuspended in saline (10 mL for each culture tube).

(a) Cis-9-octadecenoic acid-9,10-d_2 feeding

(b) Cis-9-octadecenoic acid-8,8,11,11-d_4 feeding

The same protocol was used for experiments (a) and (b). The labelled fatty acids (200 mg) in 95% ethanol (1 ml) were added to autoclaved basal medium A (10 L) containing Tween 40 (10 g). Two culture tubes containing the complete medium (10 mL) were inoculated with a saline cell suspension (0.05 mL), and the cultures allowed to grow for 24 h. The resulting culture (4 mL) was used to inoculate 3 x 500 mL Erlenmeyer flasks containing identical medium (150 mL). After 24 h of growth, each culture was used to inoculate identical medium

(3.25 L), each contained in 3 x 5 L culture flasks. Growth was allowed to continue for 36 h, after which the cells were harvested and the fatty acids extracted. The total amount of fatty acids obtained in the oleic acid-9,10-d_2 feeding was 18 mg. The relative proportions of the three major fatty acids were: oleic acid (C_{18}), 8%; palmitic acid (C_{16}), 58%; dihydrosterculic acid (C_{19}), 34%. The total fatty acids amounted to 10 mg in the oleic acid-8,8,11,11-d_4 feeding. The relative proportions of the three fatty acids were: C_{18}, 12%, C_{16}, 43%, C_{19}, 45%. The above data were derived from the chromatograms of the preparative H.P.L.C. runs, using standard samples for calibration purposes.

(c) Feeding experiments using 9-methyl-10-hydroxyoctadecanoic acid and 10-methyl-9-hydroxyoctadecanoic acid.

The title compounds (2 mg) in 95% ethanol (1 mL) were added separately to autoclaved medium A (100 mL) containing Tween 40 to make a final concentration of 20 mg/L (acid) and 1 g/L (Tween). Several culture tubes containing the above fatty acid medium (10 mL) and a similar medium containing oleic acid (20 mg/L) were inoculated with a saline cell suspension (0.05 mL). After 48 h, essentially no growth had occurred in the hydroxyacid containing media, while good growth was obtained with the oleic acid-containing medium.

(d) Feeding experiments using a 1:1 mixture of 9-exomethyleneoctadecanoic acid and 10-exomethyleneoctadecanoic acid

The title compounds were added along with Tween 40 to

Medium A to make a final concentration of 15 mg/L (acid) and 1 g/L (Tween). After autoclaving, this medium along with an oleic acid containing medium (20 mg/L) was inoculated in the usual manner. Good growth was obtained in both cases after 24 h, and the cells were harvested and the fatty acids extracted. G.C. analysis of the methyl esters (10' x 1/8" I.D., S.S., 3% OV 225 on Chrom W-HP (80/100), isothermal analysis at 190°C) showed that no appreciable cyclopropane fatty acid production had occurred although the exomethylene compounds were incorporated into the lipid fraction.

(e) Methionine-methyl-d_3 and biotin feeding

A filter sterilized solution of methionine-methyl-d_3 was added along with biotin to autoclaved medium B to make a final concentration of 100 mg/L (methionine) and 10 µg/L (biotin). The complete medium (10 mL) was inoculated from a stab culture. After 24 h of growth, aliquots of this culture (0.1 mL) were used to inoculate the same medium (5 x 10 mL). After 24 h of growth, each culture was used to inoculate identical medium in 5 x 500 mL Erlenmeyer flasks (200 mL each). After 36 h, only 2 flasks had grown out properly. The reason for this was not determined. The cells from all flasks were harvested and the fatty acids extracted. The total fatty acids amounted to 5.0 mg. The relative proportions of each fatty acid were: C_{18}, 11%, C_{16}, 48%, C_{19}, 41%.

(f) Methionine-methyl-d_3 and oleic acid feeding

Methionine-methyl-d_3 (120 mg, filter sterilized) was added to autoclaved medium B (1.2 L) containing oleic acid (24 mg) and Tween 40 (2.2 g). The complete medium (16 x 10 mL) was inoculated with a saline cell suspension (0.05 mL). After 24 h, these cultures (2 x 10 mL) were used to inoculate each of 8 x 500 mL Erlenmeyer flasks containing identical medium (125 mL each). The cells were harvested after 24 h of growth and the fatty acids extracted. The total fatty acid content of the cells was 20 mg. The relative proportion of each fatty acid was: C_{18}, 7%, C_{16}, 54%; C_{19}, 39%.

(g) Methionine-methyl-d_3 and biotin feeding

Methionine-methyl-d_3 (100 mg) was added to medium B (1015 mL) along with biotin (10 µg). After filter sterilization, the complete medium (14 x 10 mL) was inoculated with a saline cell suspension (0.05 mL) and allowed to grow for 24 h. The resulting cultures (2 x 10 mL) were used to inoculate 7 x 250 mL Erlenmeyer flasks containing identical medium (125 mL). 24 h later, 3 flasks were harvested and after 64 h, the remaining flasks were harvested and the fatty acids extracted. The cells from the 24 h culture contained 5.3 mg of total fatty acid. The relative proportion of each fatty acid was C_{18}, 8%, C_{16}, 38%, C_{19}, 54%. The cells from the 64 h culture contained 9.6 mg of total fatty acid. The relative proportion of each fatty acid was: C_{18}, 7%; C_{16}, 36%; C_{19}, 57%.

(h) Feeding experiments using methionine-methyl-d_3 and either biotin, oleic acid or cis-vaccenic acid

Basal medium B (2 L) was prepared and to 1600 mL was added Tween 40 (1.6 g) and to the remaining 400 mL was added biotin (4.0 µg). The media were distributed among 200 culture tubes in 10 mL portions, and then autoclaved. Cis-vaccenic acid (16 mg) in 95% ethanol (1 mL) was added in equal portions to 80 culture tubes containing the Tween 40 containing medium. The same procedure was carried out using oleic acid. L-Methionine-methyl-d_3 (210 mg in 21 mL of water) was filter sterilized separately and 0.1 mL aliquots were added aseptically to each culture tube. Each culture tube was then inoculated with a saline cell suspension (0.05 mL, a Klett reading of 360 was recorded for this inoculum). After 24 h, good growth was obtained in the biotin-containing medium, but only poor growth resulted in both fatty acid-containing media. The cells were harvested and the fatty acids extracted. The total amount of fatty acids obtained from the cells grown on biotin was 9.7 mg (C_{18}, 40%; C_{16}, 37%; C_{19}, 23%); from the cells grown on oleic acid, 5.0 mg (C_{18}, 21%; C_{16}, 53%; C_{19}, 26%); from the cells grown on vaccenic acid, 10.3 mg (C_{18}, 35%; C_{16}, 53%; C_{19}, 12%).

(i) Lactobacillic acid-19-d_2 and biotin feeding

Synthetic lactobacillic acid-19-d_2 (10 mg), Tween 40 (0.5 g) and biotin (5 µg) were added to medium B (500 mL).

The final medium was distributed in 10 mL portions among 50 culture tubes and autoclaved. One culture tube was inoculated with a saline cell suspension (0.05 mL) and allowed to grow for 24 h. The resulting cells were then harvested, washed with saline (2 x 10 mL) and after suspension in saline (10 mL), a 0.5 mL aliquot was used to inoculate the remaining 49 culture tubes. After 22 h of growth, the cells were harvested and the fatty acid (17 mg) extracted. The proportion of each fatty acid was: C_{18}, 2%; C_{16}, 62%; C_{19}, 36%.

(j) Fatty acid composition vs. Growth curve study (medium A)

A fatty acid composition vs. growth study was carried out using Medium A (600 mL), supplemented with biotin (6.0 µg). The pH of the medium was adjusted to neutrality from 5.4 with KOH. The medium was placed in 4 x 250 mL Erlenmeyer flasks (145 mL each) after filter sterilization. A saline cell suspension (1 mL, Klett reading, 475) was used to inoculate each flask). At regular time intervals, 4 x 2.5 mL aliquots were withdrawn from each flask. Two aliquots were combined to give 2 turbidity measurements and all 4 aliquots were combined to give an average pH reading. The fatty acids were extracted according to procedure II(b).

(k) Fatty acid composition vs. Growth curve study (Medium B)

Methionine-methyl-d_3 (14.5 mg) and biotin (1.45 µg) were added to medium B (145 mL) and the complete medium filter

sterilized into a 250 mL Erlenmeyer flask. An aliquot (10 mL) of the above medium, without methionine, was filter sterilized separately into a culture tube. A saline cell suspension (1 mL, Klett reading of 475) was used to inoculate the flask and another aliquot (0.07 mL) of the same suspension used to inoculate the culture tube. Growth was monitored by withdrawing 5 or 10 mL aliquots and measuring pH and turbidity. No growth occurred in the 10 mL culture.

(l) Large scale version of Experiment (k)

Methionine-methyl-d_3 (225 mg) and biotin (22.5 µg) were added to medium B (2.25 L). After filter sterilization, the medium was distributed in 145 mL portions among 15 250 mL Erlenmeyer flasks. The flasks were inoculated with a saline cell suspension (1 mL, Klett reading of 490). At 13.5 h, six flasks were harvested, after measuring pH and turbidity. The cells were dried and weighed (598 mg) and the fatty acids extracted by procedure II(a). The total fatty acid content was 4.7 mg (C_{18}, 51%; C_{16}, 37%; C_{19}, 12%). At 24 h, the same procedure was carried out with another 4 flasks. The total fatty acid content was 20.7 mg from 721 mg of cells (C_{18}, 27%; C_{16}, 41%; C_{19}, 32%). The remaining flasks did not grow out well and the contents were discarded.

(m) Intermolecular Isotope Effect Study

L-Methionine (49.475 mg), L-methionine-methyl-d_3 (49.460 mg) and biotin (10 µg) were dissolved in medium B (1 L). The

complete medium, after filter sterilization, was distributed among 14 culture tubes (10 mL each) and among 7 x 250 mL Erlenmeyer flasks (125 mL each). A saline cell suspension (0.05 mL) was used to inoculate each of 14 culture tubes. After 24 h of growth, the resulting cultures (2 x 10 mL each) were used to inoculate each of the 250 mL flasks. The cells were allowed to grow for 24 h, after which they were harvested and the fatty acids extracted. The total fatty acid content was 9.3 mg (C_{18}, 9%; C_{16}, 34%; C_{19}, 57%).

(n) Intramolecular Isotope Effects and Methionine/Formate Feedings

Medium B (2 L) containing biotin (20 µg) was supplemented with the components listed below, filter sterilized and distributed in 145 mL portions among 12 x 250 mL Erlenmeyer flasks (A_1-A_5, B_1-B_5, C_1, C_2). 130 mL of Medium was added to Flasks D and E. Flasks A_1-A_5 contained methionine-methyl-d_2 (14.5 mg each); flasks B_1-B_5 contained methionine-methyl-d_1 (14.5 mg each); flasks, C_1, C_2 contained methionine-methyl-d_3 (14.5 mg each); flask D contained methionine-methyl-d_3 (13.0 mg) and sodium formate (4.0 mg); flask E contained methionine-methyl-d_3 (6.5 mg) and sodium formate (4.0 mg). Each of the above flasks was inoculated with a saline cell suspension (1.0 mL, Klett reading of 400). Growth was monitored by measuring the pH of 5 mL aliquots. After 29 h of growth, flasks A_1-A_4, B_1-B_4, C_1, C_2 and flask E were harvested, the cells dried and weighed and the fatty acids extracted.

After 48 h of growth, the same procedure was carried out with Flasks A_5, B_5 and D. The analytical data of the above-experiment are listed in Table 13.

(o) Formaldehyde-d_2 Feeding

Formaldehyde-d_2 (3.75 mg) in water (6 mL) was prepared by the procedure outlined on p. 110, and divided into two equal portions. To one was added an equimolar mixture of tetrahydrofolic acid and 2-thioethanol (30.5 mg); a few drops of 5N NaOH were added to effect complete solution. The other portion was used without further modification. The contents of each feeding solution were added to medium B (100 mL each), which also contained L-methionine (10 mg) and biotin (1 µg). The media were filter sterilized and placed in 250 mL Erlenmeyer flasks. A saline cell suspension (0.7 mL) was used to inoculate the 2 flasks. After 80 h, no growth had occurred in the tetrahydrofolic acid-containing medium (final pH was 6.8) while substantial growth had occurred in the formaldehyde-containing medium (final pH was 4.0). The cells in the latter flask were dried, weighed (55 mg) and the fatty acids (1.3 mg) extracted). (C_{18}, 13%; C_{16}, 45%; C_{19}, 42%.)

(p) Methionine-methyl-d_3 Feeding under Aerobic and Anaerobic conditions

Medium B (1.65 L), containing biotin (1.7 µg) and methionine-methyl-d_3 (165 mg), was filter sterilized and dis-

Table 13

Analytical Data for Experiment (n)

Flask	Dry Cell Wgt (mg)	Cell Yield (mg/100 mL)	Total Fatty Acid (mg)	% C_{18}	% C_{16}	% C_{19}
A_1-A_4	640	110	14.2	27	38	35
B_1-B_4	646	111	8.1	37	36	27
C_1, C_2	308	106	5.8	29	28	43
D	175	135	2.2	18	48	34
E	232	178	3.2	35	32	33
A_5	176	121	2.5	20	40	40
B_5	177	122	1.9	21	41	38

tributed among 11 x 250 mL Erlenmeyer flasks (150 mL each). A saline cell suspension (1 mL) was used to inoculate each flask. Three flasks were fitted with 2-hole rubber stoppers and sterile argon passed through the medium for 0.5 h. The remaining flasks were fitted with disposable plugs to allow growth under aerobic conditions. The anaerobic and aerobic cultures were grown for 48 h, after which the anaerobically grown cells were harvested, dried, weighed (542 mg) and extracted to obtain the fatty acids (9.5 mg, C_{18}, 28%; C_{16}, 38%; C_{19}, 34%).

To isolate the methionine from the aerobically grown cells, the following procedure was used. The cells were washed with water (2 x 150 mL), and resuspended in water (50 mL) in a r.b. flask. The mixture was heated to boiling over 0.5 h and kept at 100°C for a further 10 min, with stirring. The mixture was cooled and centrifuged to obtain a pale yellow supernatant. The cells were dried, weighed (994 mg) and extracted to obtain the fatty acids (9.5 mg, C_{18}, 29%; C_{16}, 35%; C_{19}, 36%). The supernatant was reduced in volume to 10 mL and an aliquot (100 µl) subjected to automatic amino acid analysis to determine the methionine present.

IV Isolation of Methionine

Automatic amino acid analysis showed that there were ca. 950 µg of methionine present in the 10 mL extract of

994 mg of dry cells. Thus, the intracellular pool of methionine was 0.65 µmole per 100 mg of dry cell weight. To an aliquot of the extract (2.5 mL) was added sulfosalicylic acid to precipitate the protein present in the sample. The sample was concentrated to 500 µl, filtered through a 20 µm Millipore filter and ca. 0.1 µcurie of methionine-methyl-^{14}C (2.µg) was added. The sample was injected onto the automatic amino acid analyzer and the effluent collected at the column exit in 2.5 mL fractions, using a Gilson automatic fraction collector. Each fraction was checked for radioactivity by counting an aliquot (50 µl) which had been dissolved in A.C.S. aqueous counting scintillant. The elution profile is shown in Figure 27.

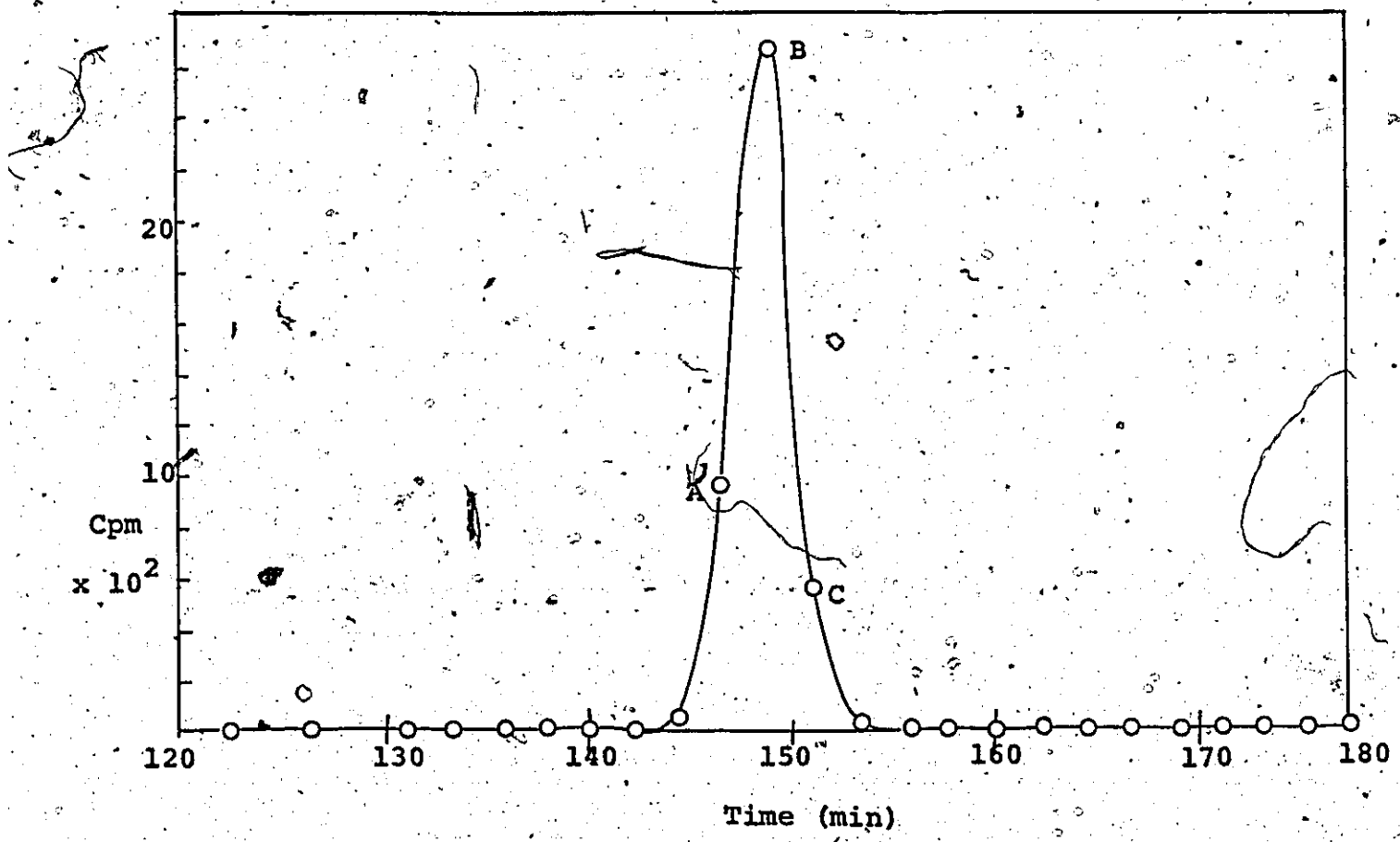


Figure 27. Isolation of Methionine from Automatic Amino Acid Analyzer

It can be seen that all of the radioactivity was contained in 3 fractions, collected at a retention time of ca. 148 min.

The three fractions were individually cleaned up to remove lithium citrate buffer. The procedure used was as follows. Dowex 1 X 8 - 200 (100-200 mesh) ion exchange resin (6 g) was washed with 2M NaOH (50 mL) by stirring for 15 min, filtered and washed with water (100 mL). (All water used in this procedure was deionized and passed through a charcoal filter.) The process was repeated with 2.5M HCl and the cycle repeated once more. Two more cycles were carried out with warm 2M NaOH (50°C) and 2M acetic acid. The resin was then suspended in water and a 1.4 cm x 7 cm column made up. The column was washed with 2M NaOH at 45°C (25 mL). (The NaOH solution was made up in CO_2-free water and the washing procedure was carried out under a KOH drying tube.) The column was washed with water (20 mL) until the pH of the effluent was ca. 7.0. The 2.5 mL fraction containing methionine and buffer was then placed on the column and the column washed with water (20 mL) until the effluent was neutral. Acetic acid (1 M) was added to elute the methionine and one 50 mL fraction collected. The column was regenerated by washing with 1M HCl (20 mL) and then water (20 mL). The 3 methionine-containing fractions were evaporated, made up to 2.0 mL in water and an aliquot (10 µl) counted. It is evident from Table 14 that all the radioactivity had been recovered from the column.

Table 14

Recovery of Radioactive Methionine After Clean-Up

Fraction 1	Radioactivity Before Clean-up (cpm)	Radioactivity After Clean-up (cpm)	%
A	4.97×10^4	5.0×10^4	22
B	1.36×10^5	1.45×10^5	64
C	2.8×10^4	3.14×10^4	14

By T.L.C. (70% ethanol, ninhydrin spray detection), it was estimated that only 1-10 µg of methionine was present in all the fractions combined. This was about 25x less than anticipated and may have been caused by decomposition of methionine in the extract, which had been kept in the dark for 4 months at 5°C.

Fraction B was evaporated under nitrogen in a 2.0 mL reactivial which had previously been treated with a 5% dimethyldichlorosilane in toluene solution to render the surface hydrophobic. The sample was dried in vacuo for several hours. A mixture of acetonitrile/BSTFA (1:3, 100 µL) was added via syringe and the mixture heated at 100°C for 10 min and the sample analyzed by GC/MS using the following GC conditions: 3% OV-1 on Gas Chrom Q (110/120), 6' x 2 mm I.D., glass, temperature programmed from 50°C-250°C at 10°/min. The M-117 ion (loss of carbosiloxy) from the bis TMS derivative of methionine was monitored at m/e 176-179 and the deuterium content calculated from the ion intensities.

D Estimation of Deuterium Content by Mass Spectrometry

(I) Initially a CEC Model #21-110B, double-focussing mass spectrometer at 70 ev was used. For determining the deuterium content of fatty and methyl esters, approximately 500 µg of sample were required and a source temperature of not less than 100°C was necessary, in order to obtain an ion beam of sufficient intensity and duration. During the latter part of

this work, a V.G. Micromass 7070F double-focussing instrument at 70 ev and coupled to a Varian model 3700 GC, was used. Approximately 1 µg of sample was injected onto the appropriate GC column and the appearance of the sample monitored on an oscilloscope display. The ions were repetitively scanned once per second throughout the entire GC peak.

II Source of Error

K. Biemann (83) has provided a thorough discussion of the possible sources of error in measuring isotope content by MS. Those of particular relevance to this work are discussed below.

a) M + 1 peaks due to ion-molecule collisions were a serious problem in the MS of the olefinic and cyclopropyl fatty acid methyl esters; at higher pressures, these compounds gave appreciable M + 1 peaks (10% of M^+). The calibration spectra of the non-labelled compounds were run at approximately the same sample pressures as the labelled materials, and a $M + 1/M^+$ ratio of 0.28 and $M + 2/M^+$ ratio of 0.05 was used for calculating deuterium contents of compounds run on the CEC instrument. In the GC/MS method, the M + 1 peak was fairly constant, as long as $\leq$ 1 µg of sample was used (e.g., for the C_{19} cyclopropyl methyl esters, the theoretical $M + 1/M^+$ ratio due to ^{13}C, ^{2}H and ^{17}O is 0.22298 (84), the observed value was 0.235 ± 0.01).

b) Changes in sample pressure during scanning of the ions,

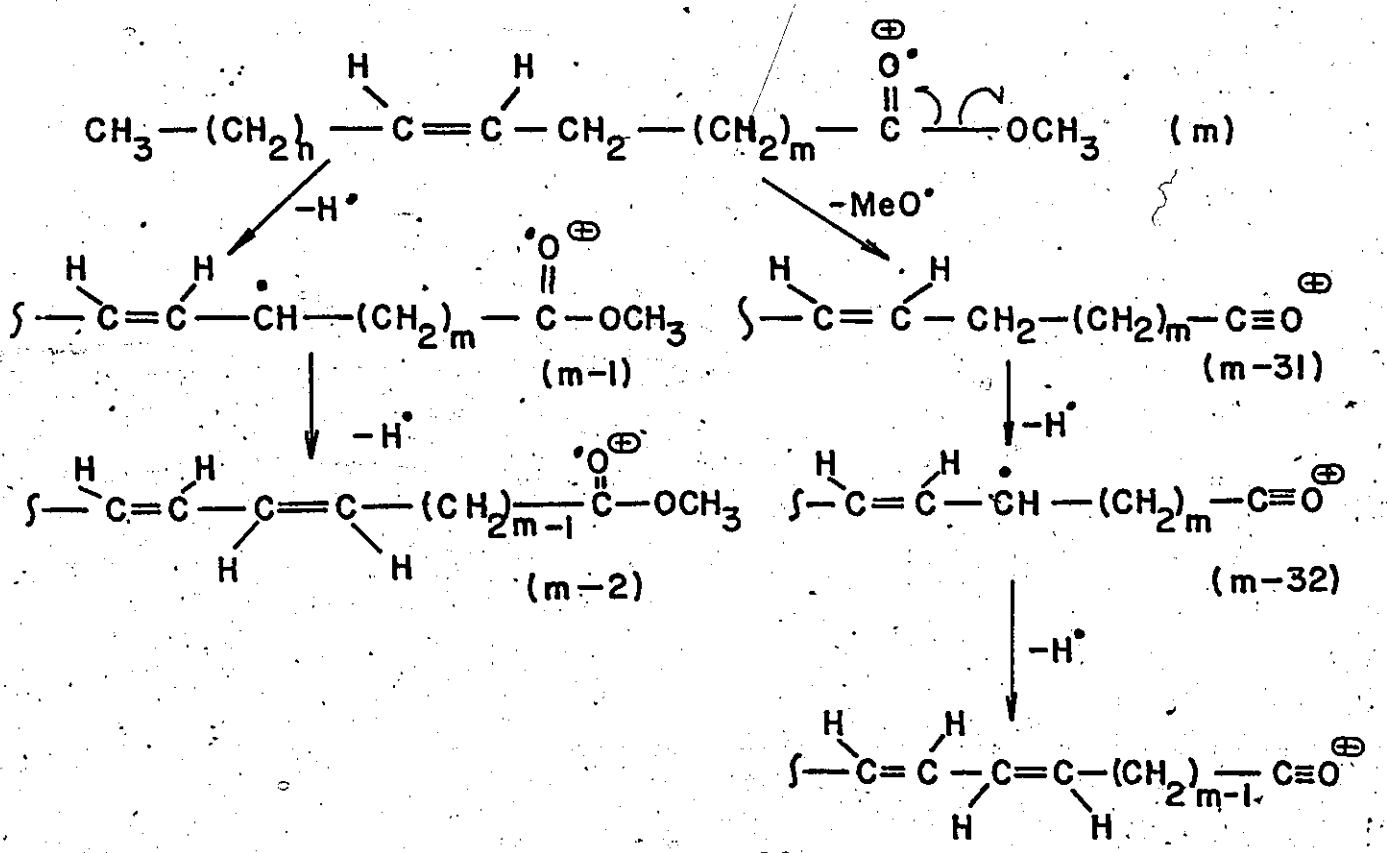
might lead to considerable error. The CEC instrument has a slow scan speed and thus it was important that a constant sample vapour pressure be established before scanning. In the GC/MS method, the scan rate was fast enough to accomodate the change in ion beam intensity during elution of the GC peak. This was confirmed by running the same sample twice and comparing the total ion intensity obtained by scanning the ions of interest up the mass scale and the results obtained by scanning down the mass scale. For a deuterated cyclopropyl compound, scanning down gave the following ion intensities: M^+ (100), M + 1 (34.8), M + 2 (86.1). Scanning up gave the following ion intensities: M^+ (100), M + 1 (35.5), M + 2 (86.1). Thus, the results are certainly within experimental error, estimated to be ± 1 for any measured intensity.

c) Unsaturated and cyclopropyl fatty acid methyl esters gave rise to small M-1 (2% of M^+) and M-2 (4% of M^+) peaks which could not be reduced in intensity by lowering the electron energy. They probably arise by the following pathway (see Scheme 26, the origin of the M-31, M-32 and M-33 peaks are also shown).

The cyclopropyl fatty acid methyl esters ring cleave and rearrange to give mass spectra identical with those of unsaturated fatty acid methyl esters except for the additional methylene unit (49). The M-1 and M-2 peaks were corrected for in the calculation of deuterium content (vide infra). The M-1 and M-2 peak intensities varied somewhat

with source temperature, so it was important that the calibration compound be run at the same temperature as the labelled compounds.

d) Finally, another source of error, important only in the GC/MS work, was isotopic fractionation of the sample on the GC column. Thus, the entire GC peak was scanned and the peak intensities integrated to obtain an accurate assessment of deuterium content. The fractionation of a deutero-labelled cyclopropyl fatty acid methyl ester is demonstrated in Figure 28.



Scheme 26

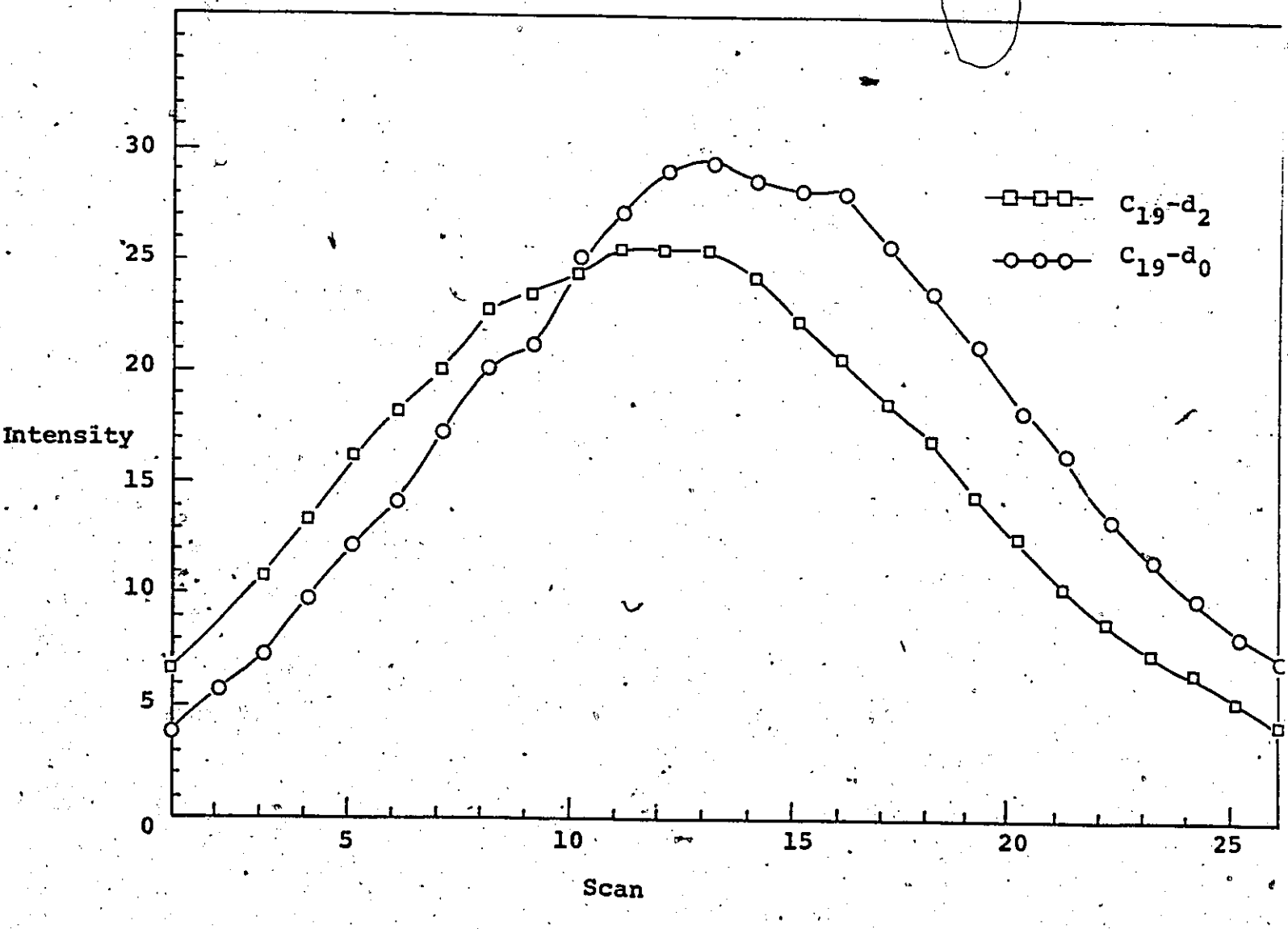


Figure 28. Fractionation of dideuterio and non-labelled methyl lactobacillate.

(III) Method of Calculation of Deuterium Content

The following discussion applies equally well to the MS of labelled olefinic fatty acid methyl esters, regardless of the position of the double bond. The distribution of deuterium could be calculated by solving two sets of simultaneous equations, one for the M^+ ion, and one for the M-32 ion (the coefficients were determined from the MS of a calibration (non-labelled) compound).

For the analysis of the M^+ ion, the following equations were solved:

$$100\ d_0 + 1.5\ d_1 + 3.0\ d_2 = \text{Intensity at m/e 310}$$
$$28\ d_0 + 100\ d_1 + 1.5\ d_2 = \text{Intensity at m/e 311}$$
$$5.0\ d_0 + 28\ d_1 + 100\ d_2 = \text{Intensity at m/e 312}$$

The following three simultaneous equations were solved for the analysis of the M-32 ion:

$$100\ d_0 + 3\ d_1 + 0\ d_2 = \text{Intensity at m/e 278}$$
$$50\ d_0 + 100\ d_1 + 5\ d_2 = \text{Intensity at m/e 279}$$
$$8\ d_0 + 50\ d_1 + 100\ d_2 = \text{Intensity at m/e 280}$$

The three simultaneous equations were solved using a Texas Instrument Programmable 58-C calculator. The calculated deuterium content from analysis of the M^+ and M-32 ions agreed to within ± 1% for each deuterated species expressed as a percent. Running the same compound on the CEC and VG 7070 mass spectrometer, yielded the same (within experimental error) calculated deuterium content: on the CEC; d_2, 79.9%; d_1, 16.0%;

d_0, 4.1%; on the VG 7070; d_2, 79.9%; d_1, 15.9%; d_0, 4.2%.

IV Deuterium Content of Cyclopropyl Fatty Acid Methyl Esters, Labelled at C-19

Of particular importance in this work, was the analysis of methyl dihydrosterculate and methyl lactobacillate, deuteriated in the bridgehead position. In particular, an accurate assessment of the d_1 content in these samples was required. To this end, synthetic dihydrosterculate-19-d_2 was prepared by a procedure identical with that outlined on p. 107, except for the use of methylene-iodide-d_2 (d_2, 98%; d_1, 2%, d_0, 0%). The intensities of the M^+ ions and the M-32 ions of both labelled and non-labelled methyl dihydrosterculate are outlined in Table 15.

Table 15

Ion Intensities of Methyl Dihydrosterculate-d_2 and Methyl Dihydrosterculate-d_0

	19-d_2 Fatty Acid	d_0 Fatty Acid
M^+	1.00	100
M-1	4.5	1.5
M-2	3.0	3.0
M-31	48	50
M-32	100	100
M-33	7.8	1.0

The increase in the M-1 ion intensity of the 19-d_2 compound can be accounted for (within experimental error) by the d_1 content of the CD_2I_2 used to prepare this compound.

However, the increase in the M-33 ion intensity arises from both the above mentioned factor and also by random loss of deuterium in lieu of hydrogen from the M-31 ion (5.7%, neglecting isotope effects).

SUMMARY

The work presented in this thesis provides mechanistic information on the biosynthesis of cyclopropane fatty acids. Efficient methods were developed for the preparation of oleic acid-9,10-d_2 and oleic acid-8,8,11,11-d_4 with high stereochemical purity and high deuterium content. When these labelled olefins were cyclopropanated by L. plantarum, no scrambling or loss of label occurred. One of the implications of these results is that biological cyclopropanation does not involve activation of the olefin at the allylic position.

Administration of methionine-methyl-d_3 to L. plantarum resulted in a mixture of d_1- and d_2-cyclopropane fatty acid, with all the deuterium at the bridgehead carbon. The amount of d_1-material was shown to increase with cell growth. It was also shown that the single deuteron in the d_1-species occupied the exo and endo bridgehead positions to the same extent.

By comparing the deuterium contents of cyclopropyl fatty acids derived from methionine-methyl-d_2 with that from methionine-methyl-d_3, it could be calculated that at least one-third of the cyclopropyl product was derived from a d_2-methyl species in the latter experiment.

The origin of this d_2-methyl species was studied extensively. It was determined that the cyclopropyl compound

does not undergo reversible ring fission once it leaves the active site of cyclopropane synthetase. Exchange at the methionine stage was ruled out by showing that one-carbon units such as formaldehyde and formate are not incorporated into the cyclopropane ring to any appreciable extent. Also, methionine-methyl-d_3 was reisolated from the bacterial intracellular pool and shown to be devoid of d_1- or d_2-species. Thus, it was concluded that the exchange process was probably occurring on the active site of the enzyme, by reversible cyclopropane ring formation. The results of this work suggest that the exchange which has been observed before in other methylations of isolated double bonds is occurring by the same mechanism.

An intermolecular primary deuterium isotope effect of 1.07 ± 0.04 was measured for the proton abstraction step in cyclopropane formation. This result was interpreted to mean that proton abstraction is not the rate limiting process in this reaction. A minimum intramolecular primary deuterium isotope effect using methionine-methyl-d_1 was measured to be 3.2 ± 0.5 in fair agreement with the isotope effect (2.8 ± 0.2) obtained for cycloartenol formation, a reaction involving a 1,3-elimination process to form a cyclopropane ring.

A model for the active site of cyclopropane synthetase is presented in order to account for the reversibility of cyclopropane ring formation. On the basis of the above results, it is believed that the mechanism of the biological

cyclopropanation reaction involves a methyl transfer to the olefin followed by a 1,3-proton elimination step.

APPENDIX I

^{13}C Chemical Shifts of Various Derivatives of Cis-9,10-Octadecenoic Acid

Carbon # \ Compound	1	2	3	4	5	6	7*
1	174.5	180.3	180.5	173.5	173.6	174.3	174.3
2	33.8	34.1	34.2	34.1	34.1	34.1	34.1
3	24.6	24.6	24.7	25.3	25.3	25.0	25.7
4	28.7-29.2	29.0-29.3	28.7-29.1	29.5-30.5	29.5-30.5	29.3-29.7	29.1
5	28.7-29.2	29.0-29.3	28.7-29.1	29.5-30.5	29.5-30.5	29.3-29.7	29.1
6	28.7-29.2	29.0-29.3	28.7-29.1	29.5-30.5	29.5-30.5	29.3-29.7	29.1
7	25.6	23.1	28.7-29.1	26.7	27.9	26.7	23.9, 29.1
8	32.5	36.1	18.8	35.1	33.8	27.9	42.9, 23.9
9	73.9	200.3	80.1	74.9	38.9	57.2	211.4, 42.9
10	73.9	200.2	80.4	38.9	74.9	57.2	42.9, 211.4
11	32.5	36.1	18.8	33.9	35.1	27.9	23.9, 42.9
12	25.6	23.0	28.7-29.1	28.0	26.9	26.7	29.1, 23.9
13	28.7-29.2	29.0-29.3	28.7-29.1	29.5-30.5	29.5-30.5	29.3-29.7	29.1, 23.9

Continued.....

APPENDIX I (Continued)

Compound / Carbon #	1	2	3	4	5	6	7*
14	28.7-29.2	29.0-29.3	28.7-29.1	29.5-30.5	29.5-30.5	29.3-29.7	29.1, 23.9
15	28.7-29.2	29.0-29.3	28.7-29.1	29.5-30.5	29.5-30.5	29.3-29.7	29.1, 23.9
16	32.4	31.8	31.9	32.4	32.4	31.9	31.9
17	22.2	22.6	22.7	23.2	23.1	22.8	22.7
18	13.9	14.1	14.1	14.4	14.3	14.1	14.1
CO_2CH_3				51.0	51.0		51.4
19				14.0	14.0		

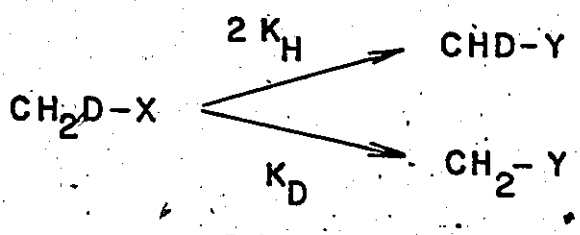
* For C-7 to C-12, the chemical shifts for the 9-oxo compound are given first.

Legend:
1. erythro-9,10-Dihydroxyoctadecanoic acid
2. 9,10-Dioxooctadecanoic acid
3. 9-Octadecynoic acid
4. Methyl 10-methyl-9-hydroxyoctadecanoate
5. Methyl 9-methyl-10-hydroxyoctadecanoate
6. Methyl cis-9-epoxyoctadecanoate
7. Methyl 9-oxooctadecanoate and methyl 10-oxooctadecanoate

APPENDIX II

A CALCULATION OF PRIMARY INTRAMOLECULAR DEUTERIUM ISOTOPE EFFECTS

For the loss of one hydrogen (deuteron) from a methyl-d_1 group, the following kinetic scheme obtains (Scheme 27):



Scheme 27

The rate of d_1 and d_0 cyclopropane formation is given by

$$\frac{d[d_1]}{dt} = 2k_H[CH_2D\text{-}X]$$

$$\frac{d[d_0]}{dt} = k_D[CH_2D\text{-}X]$$

Taking the ratio of the rates, yields the expression for the intramolecular deuterium isotope effect:

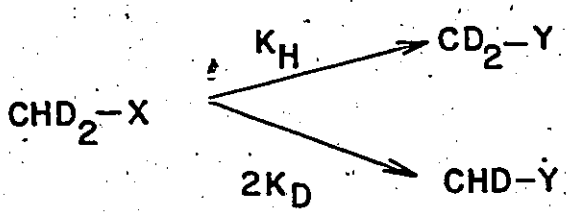
$$\frac{d[d_1]}{d[d_0]} = \frac{2k_H}{k_D}$$

Integrating yields: $\frac{d_1}{d_0} = \frac{2k_H}{k_D}$

Since $d_1 + d_0 = 1$, $\frac{\% \; d_1}{\% \; d_0} = \frac{2k_H}{k_D}$

Rearranging, $\frac{k_H}{k_D} = \frac{\% \; d_1}{2(\% \; d_0)}$

For the conversion of a methyl-d_2 group into a methylene group, the following kinetic scheme obtains (Scheme 28):



Scheme 28

The rate of d_2 and d_1 cyclopropane formation is given by

$$\frac{d[d_2]}{dt} = k_H[CHD_2\text{-}X]$$

$$\frac{d[d_1]}{dt} = 2k_D[CHD_2\text{-}X]$$

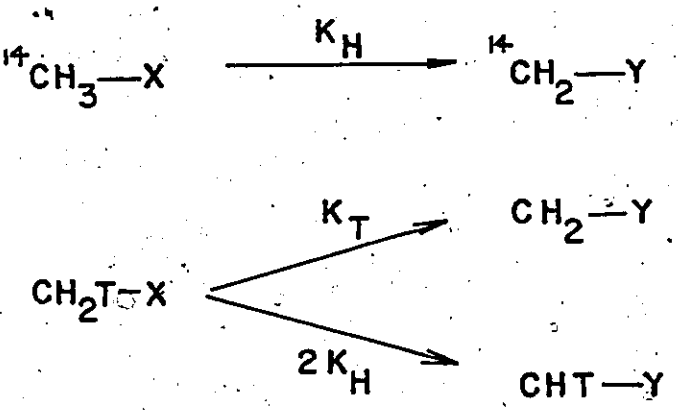
Taking the ratio of the rates, integrating and rearranging yields the expression for the intramolecular deuterium isotope effect:

$$\frac{d_2}{d_1} = \frac{k_H}{2k_D}$$

Thus, $$\frac{k_H}{k_D} = \frac{2(\%\ d_2)}{\%\ d_1}$$

B CALCULATION OF THE INTRAMOLECULAR TRITIUM ISOTOPE EFFECT FROM THE WORK OF POHL ET AL. (5)

The above workers fed a mixture of methionine-methyl-^3H, and methionine-methyl-^{14}C in the ratio of 0.427 to 1. They isolated a biosynthetic cyclopropane which had a $^3H/^{14}C$ ratio of 0.39 to 1. The following kinetic scheme applies if there is no intermolecular isotope effect (Scheme 29):



Scheme 29

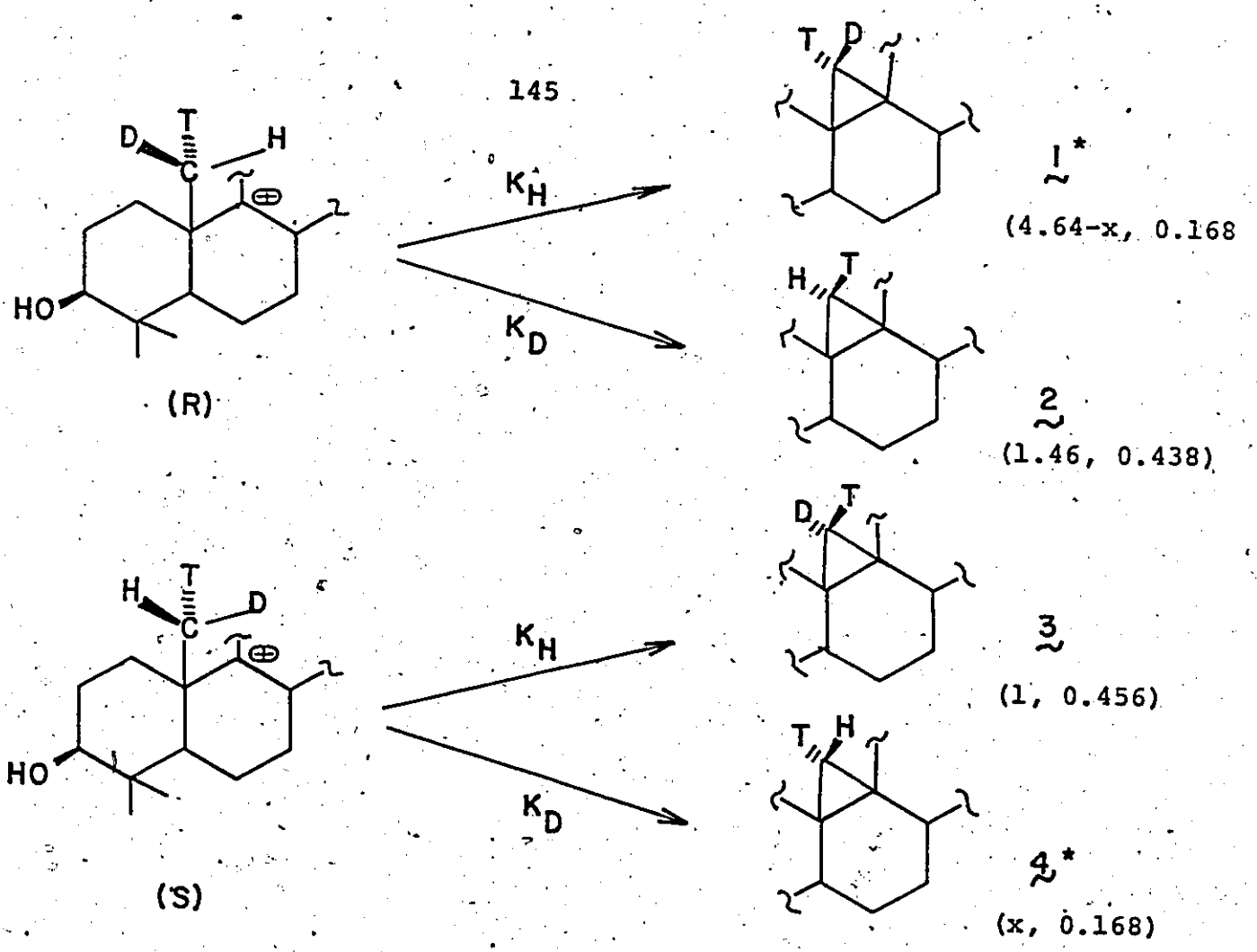
Thus $\frac{d[(CHT)]}{dt} = 2k_H[CH_2T\text{-}X]$ and $\frac{d[(CH_2)]}{\quad} = k_T[CH_2T\text{-}X]$.

Using similar arithmetic manipulations as in Appendix IIA, one arrives at an expression for $\frac{k_H}{k_T} = \frac{\%(CHT)}{2\%(CH_2)} = \frac{0.39}{0.037} \times \frac{1}{2} = 5.3$.

C CALCULATION OF THE INTRAMOLECULAR DEUTERIUM ISOTOPE EFFECT FROM THE WORK OF ALTMANN ET AL. (58)

The above workers fed chiral-methyl labelled oxidosqualene, (80% (R)-methyl and 20% (S)-methyl) which when cyclized gave the following distribution of products (see Scheme 30; the figures in parentheses represent, ^3H nmr intensities and the chemical shift in ppm).

The value of x could be calculated by assuming identical rates of formation of compounds 2 and 4. Since the (R)-methyl compound was present at four times the concentration of the (S)-methyl compound, then $x = \frac{1.46}{4} = 0.365$. Thus, the contribution of compound 1 to the intensity of the signal at 0.168 ppm was 4.64 - 0.365 = 4.275 (this represents an error of 7% from the expected value of 4.0). By taking the ratios $\frac{4.275}{1.46} = 2.93$ and $\frac{1}{0.365} = 2.74$, one arrives at an average intramolecular deuterium isotope effect of 2.84 ± 0.2.



Scheme 30

* Tritium nmr signals due to compounds 1 and 4 were not resolved.

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