

METABOLISM OF MEVALONIC ACID

BY

LACTOBACILLUS PLANTARUM

MUGHARBIL

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by

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A C K N O W L E D G E M E N T

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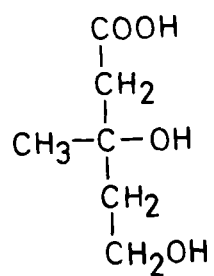
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INTRODUCTION

A. Discovery of Mevalonic Acid

The search for vitamins and their biochemical role was one of the main problems of scientists in the early thirties. The number of vitamins increased as biochemists continued to look for growth promoting factors in distiller's dried soluble, rice polishing concentrates, liver extracts and others (1). In (1946) Guirard et al. (2) demonstrated the marked effect of sodium acetate in stimulating the early growth of lactic acid bacteria. They also demonstrated the growth promoting effect of various compounds. Skeggs et al. (3) in (1956) reported that many lactobacilli such as L. acidophilus, L. bifidus, and L. bulgaries failed to grow in semisynthetic medium devoid of acetate, but grew in the presence of the above mentioned concentrates. Fractionation of the distiller's dried soluble and intensive purification yielded a compound which was proved to be the lactone of 3,5-dihydroxy-3-methylpentanoic acid (1, 3, 4, 5). This was given the trivial name mevalonic acid. The structure was proven by two unambiguous syntheses, and it was half as active as the naturally occurring one in supporting the growth of lactobacilli. The synthetic compound was later resolved into its optically active isomers. The absolute configuration and optical rotation of the biologically active isomer were established (1, 6, 7) (Figure 1.)



D-(+) mevalonic acid

FIGURE 1. D-(+) MEVALONIC ACID

B. Role of Mevalonic Acid in the Synthesis of Terpenes and Sterols.

Although several precursors of sterols were known by (1956) the nature of the biologically active isoprene unit remained unknown. As early as (1956) ^{6,8} hydroxymethylglutaric acid and other compounds were proposed as possible precursors of the isoprene unit, but these were poorly incorporated into sterols (8). The obvious structural similarity between mevalonic acid and hydroxymethylglutaric acid tempted Tavormina et al. (9) in (1956) to study the role of mevalonic acid in the biogenesis of sterols in liver homogenates. It was found that mevalonic acid was efficiently incorporated into cholesterol.

The mechanism by which mevalonic acid is incorporated into terpenes and sterols in liver homogenates, yeast, and higher plants has been well established by several workers (10). The first stage of utilization is an ATP dependent phosphorylation to mevalonic - 5 - phosphate by an enzyme which is now termed mevalonic Kinase. The mevalonic - 5 - phosphate is further phosphorylated to mevalonic pyrophosphate by another Kinase. Dehydration and decarboxylation of mevalonic pyrophosphate by one enzyme occurs with the consumption of a third mole of ATP to give Δ^3 - isopentenylpyrophosphate which is enzymatically isomerized to γ - γ' dimethyl allylpyrophosphate. (Figure 2.) These are the structural units of terpenes, sterols, carotenoids and rubber (9, 11, 12). Note that the carboxyl group of mevalonic acid is lost in the formation of Δ^3 - isopentenylphosphate. This was first utilized by Durr and Shwayri (13) for the assay of mevalonic acid

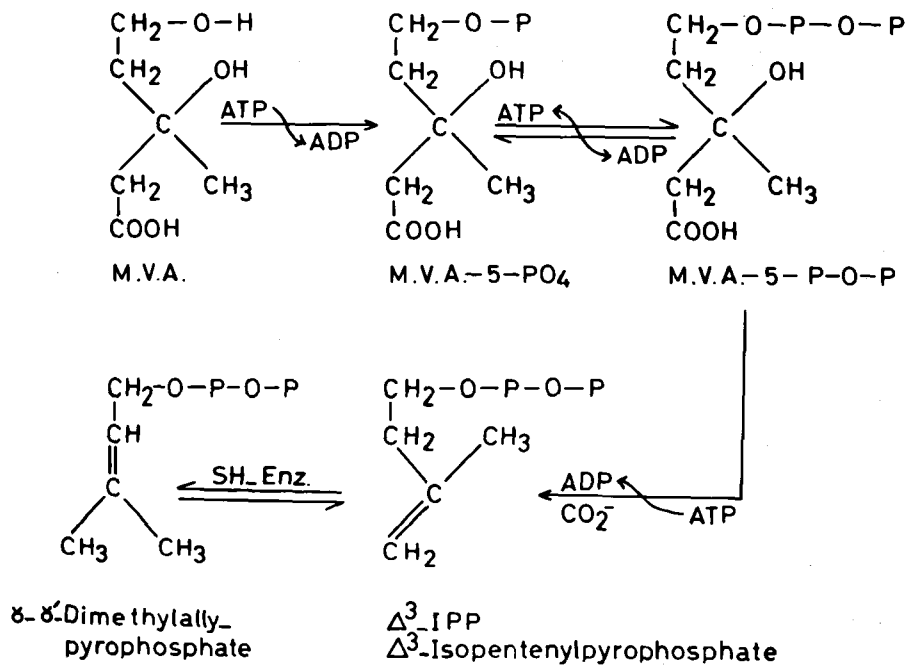


FIGURE 2. THE BIOSYNTHESIS OF THE BIOLOGICALLY ACTIVE ISOPRENE UNIT (Δ^3 - IPP)

metabolism by lactobacilli.

C. Bacterial Lipids and Metabolism of Mevalonic Acid

Studies on the lipids of lactobacilli were initiated by Crowder and Anderson (14) in the early thirties. These, and later workers investigated the components of the saponifiable lipids and succeeded in isolating among other compounds, lactobacillic acid $C_{19}H_{36}O_2$ from L. arabinosis, and L. casei (15).

Little attention was paid to the non-saponifiable fraction until (1956), when mevalonic acid was discovered as the acetate-replacing factor (3). Furthermore, the finding that mevalonic acid was the long sought precursor of sterols in animals, yeast and higher plants, raised again the question whether sterols are components of bacterial non-saponifiable lipids. It has been also reported that acetate, needed for the growth of lactobacilli in general, could be replaced by sterols and terpenes. Of particular interest was the finding that the Liebermann-Burchard positive fraction of the non-saponifiable lipids was very active in replacing acetate (2).

In spite of the above mentioned facts, application of Liebermann-Burchard reaction and digitonin precipitation to the non-saponifiable fraction isolated from L. acidophilus and L. casei derived from mevalonic acid indicated absence of sterols in these strains (16). Although cholesterol was isolated from lactobacilli it was later shown that the compound was derived from the growth medium (14, 17). Subsequent investigation of one component of the non-saponifiable fraction derived from L. casei suggested that it is a polymer of the isoprene unit with

the empirical formula $C_{100}H_{177}O_{11}$ (18).

Investigators (16, 18) working with mevalonic acid-dependent strains of lactobacilli, reported also that several nutrients were required for the incorporation of mevalonic acid into the non-saponifiable lipids. In (1964) Durr and Shwayri (13) working with mevalonic acid-independent strain of lactobacilli, L. plantarum, reported that this bacterium incorporated one isomer of DL-mevalolactone with the liberation of one mole carbon dioxide for every mole of radioactive carbon incorporated in the non-saponifiable lipids. They also reported that amino acids have no effect on this incorporation, yet glucose is required as source of energy.

Very recently Suzue et al. (19) working on mutant of *Staphylococcus* were able to identify the 5-phosphomevalonate which is involved in the biosynthesis of phytoene in the enzymatic preparation of this mutant.

The decarboxylation of mevalonic acid and the identification of 5-phosphomevalonate from *Staphylococcus* suggest that the non-saponifiable lipids are polymers of the biologically active isoprene unit.

It may be noted here that the difference between the various members of the lactobacilli for their requirement of mevalonic acid probably reflects differences in the patterns of enzymes involved in the synthesis of mevalonic acid. This was confirmed by studies on various strains of the *Mycoplasma*. It was found (20) that strains required mevalonic acid or sterols for growth were deficient in enzymes which synthesize B-hydroxy-B-methyl-glytaryl Coenzyme-A and mevalonic acid.

D. Scope of the Investigation

Bacterial lipids in general differ from the lipids of other organisms in the absence of Sterols. This conclusion rests on the negative Liebermann-Burchard test and on the absence of precipitate with digitonin. Thus more vigorous tests are needed to identify the nature of the non-saponifiable lipids of lactobacilli. This would include an elementary analysis and structural determination of the purified fractions. Towards this end we grew large amount of cells of L. plantarum. The non-saponifiable lipids of these cells were extracted by hot ethanol and fractionated by different organic solvents. The major fraction was intensively purified by chromatography. Elementary analysis and structural determinations were done on this fraction.

We have also attempted to gain insight into the metabolism of mevalonic acid by this organism by the preparation of a cell free extract which would catalyze some of the transformation of mevalonic acid into the non-saponifiable lipids. Studies on whole cells were also carried out to determine possible changes in the pattern of the various components of non-saponifiable lipids under different conditions of growth.

EXPERIMENTS AND RESULTS

I. MATERIALS AND METHODS

A. Biological Material

L. plantarum strain 8014-H₂ was obtained from the American Type Culture Collection. Stock Cultures were maintained by growing the microorganisms in tubes containing 10 ml of semisynthetic medium of Skeggs et al. (3). The tubes were kept at 30° for 24 hours, and subsequently stored at 10°. These tubes were renewed weekly.

B. Reagents

Chemicals for the growth medium were obtained from Difco, Detroit, Michigan, and Nutritional Biochemical Corp., Cleveland, Ohio. Radioactive DL-mevalolactone was obtained from the Radio Chemical Center, Amersham, England. All other chemicals were obtained from E. Merck, Darmstadt, Germany; Sigma chemical Co., St. Louis, Mo., and Shandon Scientific Co., Ltd., London.

C. Growth Conditions

Cells were grown in a 200 liter-fermentor with large inocula (10 percent). Water of the medium was heated at 85° for 24 hours, then cooled to 37°. Chemicals of the growth medium were dissolved in 12 liters of water, autoclaved, cooled, and transferred to the fermentor with 0.95 mmole of unlabelled DL-mevalolactone. Ultra-violet light was directed to the cover of the fermentor to avoid contamination. The medium could be modified by replacing casein

by yeast extract or bactopeptone. Omission of tryptophane, alanine, and pyridoxal did not affect the growth.

The medium was used 3 times. After each collection of cells, it was adjusted to pH 5.5 with sodium hydroxide pellets and sodium carbonate, heated and cooled. Additional quantities of glucose, bactopeptone and yeast extract (20 gms, 4 gms, and 4 gms/liter respectively), were added after each collection.

To check the uniformity of the cells, a Gram stain was made. Cells showed 1 percent contamination with gram negative rods. Cells were collected with a large scale separator.

In order to trace the lipids derived from DL-mevalolactone, cells were grown in 12 liters medium for 48 hours as previously described. The medium contained 1.7 mmoles of unlabelled DL-mevalolactone and 16 μ moles of DL-mevalolactone-2-C¹⁴ (14.7×10^6 counts/minute).

The labelled cells were collected by centrifugation at 4500 x g for 15 minutes then washed with distilled water until the washings were free from radioactivity. Labelled cells were mixed with the unlabelled ones, suspended in 1500 ml of distilled water and checked for radioactivity.

D. Measurement of Radioactivity

0.2 ml of sample was placed on stainless steel planchet, dried under infra-red lamp and counted on Nuclear Chicago Counter with a sensitivity of 600,000 counts/minute/u. curie and a background counting rate of 20 counts/minute.

II. LIPIDS OF L. PLANTARUM

A. Extraction of Cells

Extraction of cells with acetone-ether, or ether in the cold was not adequate. Hence the following method was developed.

1. Cells suspended in alcohol-water (3:1, v/v) were refluxed for 6 hours, cooled and filtered through a Buchner funnel. The procedure was repeated 4 to 5 times until the extracted material was exhausted from radioactivity. All the alcoholic extracts were combined and tested for activity (69×10^4 counts/minute) which corresponds to 10 percent of the added radioactivity. This calculation is based on the fact that only one isomer of DL-mevalolactone is biologically active.

2. The residue obtained from the evaporation of alcohol was exhaustively extracted with 15 - 20 ml portions of benzene, until no more radioactivity appeared in the benzene layer. The combined benzene fractions contained (55×10^4 counts/minute). The residue was next extracted with ethanol (78×10^3 counts/minute) and the extract saved. The remaining residue was water soluble and had no activity; this fraction was discarded.

3. The benzene extract (55×10^4 counts/minute) was flash evaporated to dryness and partitioned between equal volumes of n-heptane and 80 percent alcohol (figure 3.). The 80 percent ethanol (35×10^4 counts/minute) was saved. The n-heptane fraction was

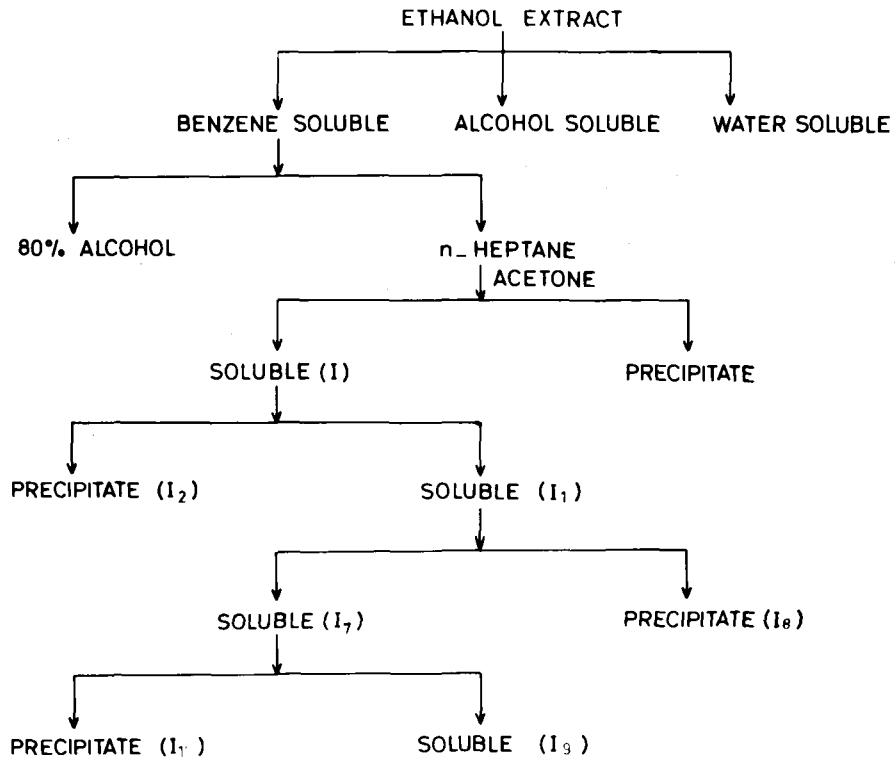


FIGURE 3. PURIFICATION OF THE ALCOHOL EXTRACT
OF CELLS

flash evaporated, and the residue was extracted with acetone. The insoluble residue (35×10^3 counts/minute) was saved. The acetone soluble fraction (I) was further purified as shown below.

4. The acetone soluble fraction was flash evaporated, resuspended in 10 ml acetone and precipitated with 3 volumes of ether. The precipitate (I_2) was saved, and the supernatant layer (I_1) was flash evaporated. The residue was redissolved in ether-acetone (1:3, v/v) and stored overnight at -20° . The resulting precipitate (I_8) was centrifuged and saved.

5. The supernatant solution (I_7) was flash evaporated and the residue was cooled overnight at -20° . The precipitate (I_{10}) was saved. The supernatant solution (I_9) contained (194×10^3 counts/minute).

A summary of these steps is shown in figure 3.

6. Chromatography of (I_9) on silicic acid: The sample (I_9) (194×10^3 counts/minute), after evaporating the acetone ether layer under a stream of nitrogen, was weighed (398.8 mg), the sample was redissolved in 3 ml ether and applied to a silicic acid column (12 x 1.4 cm). The column was developed with solvents shown in table 1. The elution pattern is shown in figure 4, which also shows that fraction (I_9) was resolved into five fractions (I_{11} - I_{14}). The percentage recovery in each fraction is indicated in table 1.

7. Thin layer chromatography of fractions (I_{11} - I_{14}): To determine whether these fractions represent a single compound, they were subjected to thin layer chromatography.

TABLE 1

CHROMATOGRAPHIC RESOLUTION OF (I₉)

Solvent	Name of fraction	Number of tubes	Total volume (ml)	Total Radio activity counts/minute	% Recovery of radio-activity
n-heptane	I ₁₁	70	350	68,400	35%
1% Ether	I ₁₂	40	200	30,000	15%
5% Ether	I ₁₃	35	175	21,000	10%
10% Ether	I ₁₄	40	200	20,000	10%
50% Ether	I ₁₅	15	75	10,000	5%
lost on the column				44,600	25%
Total				194 x10 ³	100%

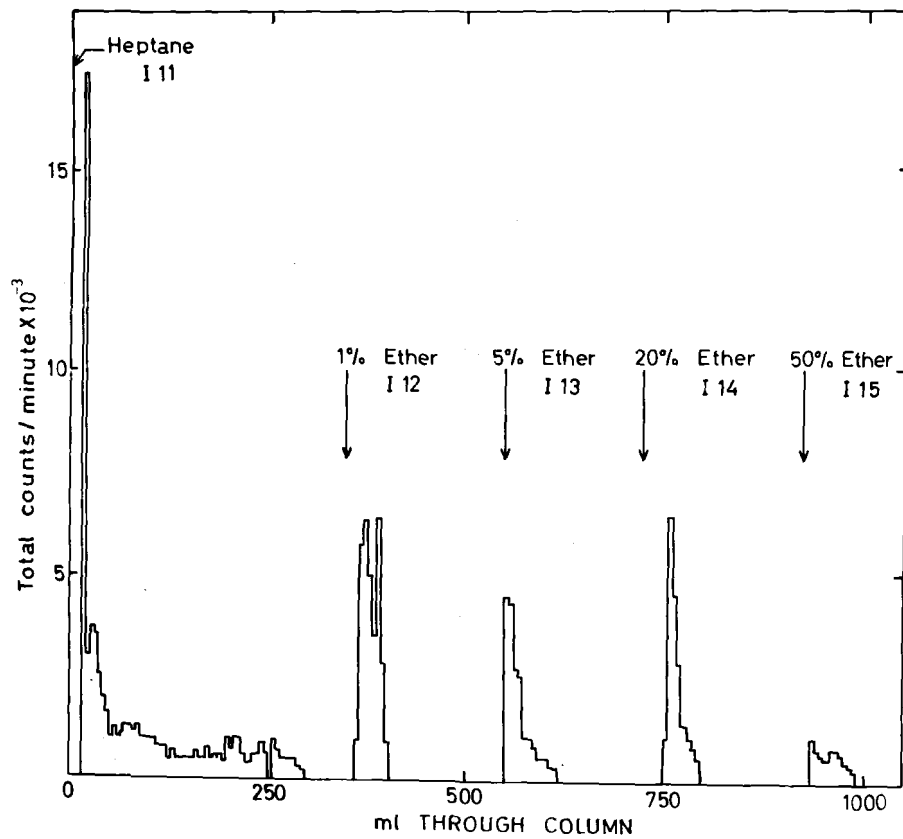


FIGURE 4. FRACTIONATION OF (I₉) ON SILICIC ACID
COLUMN CHROMATOGRAPHY

Silicic acid suitable for thin layer chromatography was suspended in distilled water in the ratio 1:2 respectively and was used for preparing plates (0.25 mm thickness). After standing at room temperature for 10 minutes the plates were heated in an oven at 110° for half an hour. Plates were stored in a desiccator (21). 10 - 20 μ gms of each of the (I) samples were applied to thin-layer plates, and the plates were developed by five different solvents as shown in table 2. Subsequently the plates were dried under/infra-red lamp, sprayed with concentrated sulfuric acid and dried at 100° . Black spots appeared on the chromatograms for all the samples, the R_f values of which are given in table 2.

It can be seen (Table 2) that more than one component is present in each fraction. Since (I_{14}) was apparently the purest of these fractions, it was chosen for further purification.

8. (I_{14}) sample was further purified with acetone-ether precipitation as described in step 4. The precipitate was saved with the other samples. The supernatant solution (12×10^3 counts/minute) was evaporated under a stream of nitrogen. The residue obtained (27 mgs) was redissolved in ether and checked for purity by thin layer chromatography using the same solvents as before (Table 2). Thin layer chromatography of (I_{14}) gave a single spot with five different solvents. The R_f values are shown in table 3.

B. Physical Properties of (I_{14})

1. (I_{14}) is a yellow, waxy compound with a pleasant odor.

2. Melting point determination: An ice-water bath was prepared.

When an aliquot of compound (I_{14}) in a small test tube was introduced

TABLE 2

R_f VALUES OBTAINED IN THIN LAYER CHROMATOGRAPHY

OF FRACTIONS (I₁₁ - I₁₄)

Sample	n-heptane	5% Ether in n-heptane	50% Ethylacetate in Hexane	Ether	Methanol
I ₁₁	0	0.970	0.787 0.160	0.764	0.450 0.775
I ₁₂	0	0.900 0.192	0.604 0.950	0.170	0.378 0.785
I ₁₃	0	0	0.80	0.87 0.80	0.68
I ₁₄	0	0	0.82 0.76	-	0.75

TABLE 3

R_f VALUES FOR (I₁₄) SAMPLE
OBTAINED BY THIN LAYER CHROMATOGRAPHY

Solvent	R _f
n-heptane	0
5% Ether in n-heptane	0
50% Ethyl acetate in hexane	0.73
Ether	0.84
Methanol	0.81

to this bath it solidified. Temperature of the bath was raised gradually. Sample melted between 20-21°.

C. Chemical Properties of (I₁₄)

1. Elementary analysis: A microanalysis of (I₁₄) dried to a constant weight, (Dr. Franz Pascher, Mikroanalytisches Laboratorium, 53 Bonn, Buschstrasse 54, Germany,) indicated that the compound contained 73.16 percent Carbon, 11.94 percent Hydrogen, 14.29 percent Oxygen, 7.66 percent (C-CH₃) and molecular weight 426 (Kofler Method). The iodine, and hydroxyl values were zero.

The empirical formula of the compound as calculated from the reported data is C₂₆H₅₁O₄ (O = 14.87 percent, H = 12.02 percent, C = 73.01 percent with a molecular weight of 427.7).

2. We have also determined the iodine value according to the British pharmacopœia (22). The determination was run on 1.17 mgs of the sample using excess of Wij's reagent. The reaction was carried in the dark for twelve hours with a potassium iodide trap. The excess iodine was titrated with 0.057 N sodium thiosulfate. The iodine value determined by this method was zero, which shows that the compound is a saturated hydrocarbon.

3. Saponification equivalent: 1.16 μmoles of the compound were saponified with 0.1 ml of 0.33 N methanolic sodium hydroxide 10 percent in a sealed tube. A blank was run under the same experimental conditions. Saponification was carried ^{out} in a boiling water bath for six hours. The excess sodium hydroxide was titrated back with 0.0301 N hydrochloric acid to phenol red end point. 1.13 milliequivalents of

sodium hydroxide were needed to saponify the sample. This test shows that the compound is a monoester with a saponification equivalent of 95.

4. Colorimetric determination for esters: Since the compound under study contains oxygen, it became of interest to investigate the nature of the oxygen containing groups.

Colorimetric determination for esters was run in duplicate using 1.17 μ moles and 2.5 μ moles. These samples were dissolved in 3 ml alcohol:ether mixture (3:1, v/v). 0.5 ml of 3.5 N sodium hydroxide and 0.5 ml of 2 M hydroxylamine hydrochloride solution were added to the sample. The tubes were stoppered and allowed to stand for 20 minutes at room temperature. After this period 0.6 ml of hydrochloride acid solution (one part of concentrated hydrochloride acid d = 1.18 diluted with two volumes of water) were added, followed by 0.5 ml of 0.37 M ferric chloride solution in 0.1 N hydrochloride acid. The tubes were mixed again and the color developed was measured at 525 m μ (23). Absorbancies of 1.17 and 2.5 μ moles of (I_{14}) were 0.51 and 0.65 respectively, which is equivalent to an optical density of 0.25/ μ mole of (I_{14}). The results of this experiment were compared with three different standards, triacetin, methylaurate, ethylolate. The optical density of 1 μ -equivalent of each of these compounds is 0.25, 0.30, 0.28 respectively. This suggests that I_{14} is a monoester.

5. Methoxy group determination: Since oxygen could not be quantitatively accounted for, we investigated the possible presence of ether linkages.

Zeisel alkoxy method (24) was run on the compound for the qualitative determination of methoxy groups. A sample of 1-2 mgs was treated with one ml of glacial acetic acid and one ml of hydroiodic acid 57 percent (sp. gr = 1.7). Upon heating in an oil bath 120-130°, the liberated alkyl halides gave a positive vermillion color with mercuric nitrate. The same test was run on the saponification products of the compound. The ether soluble fraction after being evaporated under a stream of nitrogen to remove traces of ether gave a positive reactions, while the aqueous layer gave a negative one.

6. Absorption spectrum of (I₁₄): In view of the fact that the iodine value is zero, the compound should not have any significant absorption in the ultraviolet region of the spectrum. The absorption spectrum of the compound was determined using Beckmann DU-spectrophotometer. Alcohol was used as a solvent. As shown in figure 5 (I₁₄) the compound (I₁₄) showed no well defined absorption bands in ultra-violet, although general absorption occurred beginning at about 270 mμ and extending in increasing degree to the lower limit of instrumental response.

7. Infra-red spectrum of the unknown compound was determined using Parkin-Elmer infra-red spectrophotometer (Model 237). A solution of (I₁₄) in ether was applied at the center of a sodium chloride crystal. The ether was evaporated under a stream of nitrogen and the dry sample was scanned between 650-4000 cm⁻¹.

It can be seen (Figure 6) that peak maxima are present at 3445 cm⁻¹; 1750 cm⁻¹; 2860 cm⁻¹; 2930 cm⁻¹; 1470 cm⁻¹; 1380 cm⁻¹; 970-1050 cm⁻¹; 1100 cm⁻¹; 1200-1250 cm⁻¹; and 1175 cm⁻¹.

Several spectra of authentic terpenes as squalene, farnesol, geraniol, nerolidol and linalol were determined under the same experimental conditions. The curves of some of these are shown in figure 6.

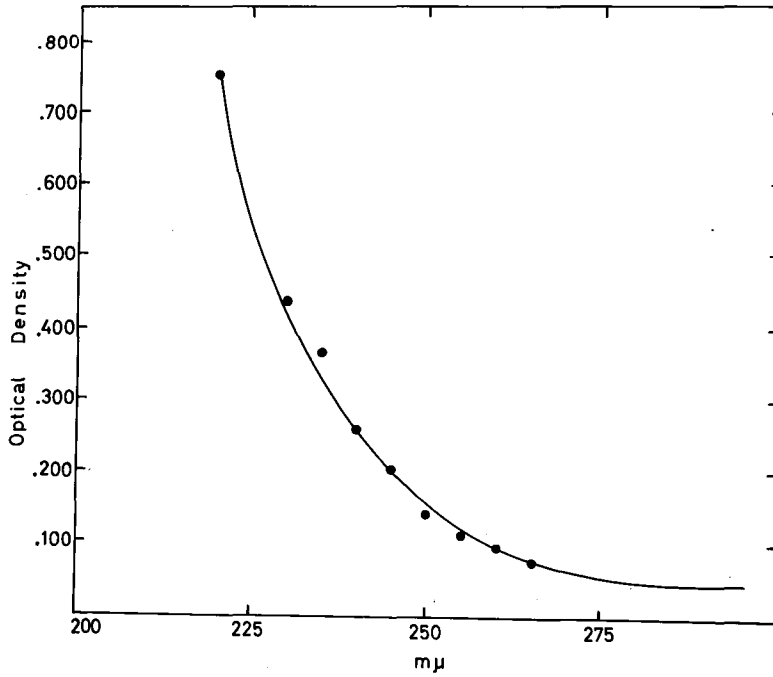


FIGURE 5. THE SPECTRUM OF (I₁₄) IN
ABSOLUTE ALCOHOL

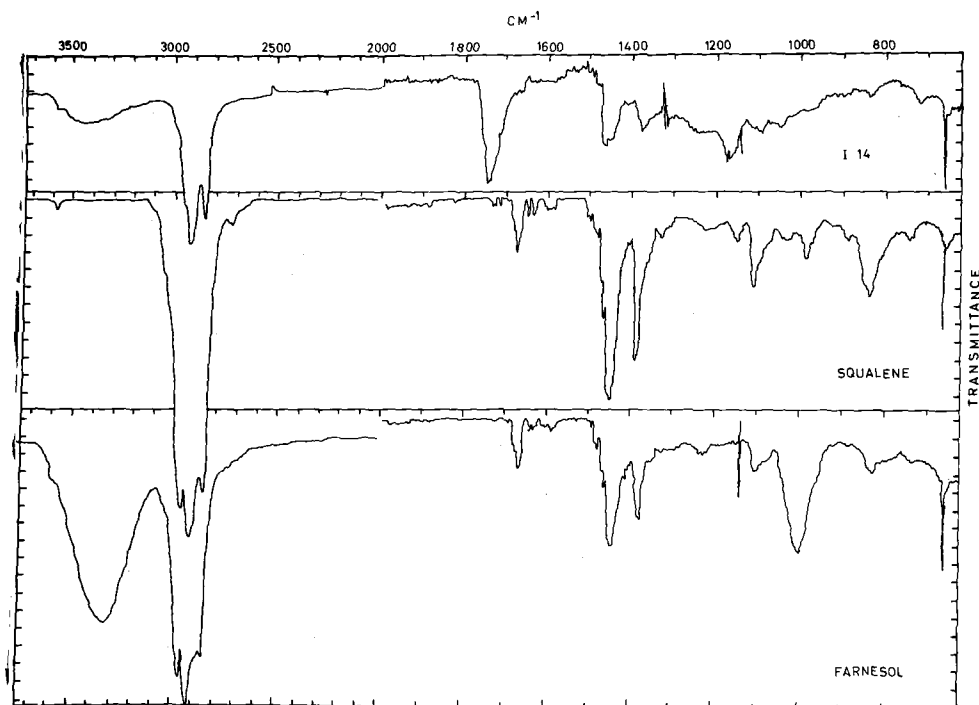


FIGURE 6. INFRARED SPECTRA OF (I₁₄),
SQUALENE AND FARNESOL

III. STUDIES ON WHOLE CELLS

A. Effect of oxygen on the growth of *L. plantarum*, and the incorporation of mevalonic acid into non-saponifiable lipids

In view of the fact that some of the non-saponifiable lipids of lactobacilli are oxygenated products as shown in this thesis and by Throne and Kodicek (16), the possibility that the synthesis of such compounds required molecular oxygen was investigated. Durr and Shwayri (13) have shown that under an atmosphere of nitrogen or carbon dioxide, the synthesis of non-saponifiable lipids by resting cell suspensions of *L. plantarum* was inhibited by 20-50 percent of that under aerobic conditions. Therefore it became of interest to determine whether anaerobic conditions also affected the extent of growth and the pattern of synthesis of various fractions of non-saponifiable lipids.

1. Two batches of cells were grown in a 24 liter medium containing one ml of mevalolactone-2-C¹⁴ (16.5×10^6 counts/minute/15 umole) each for 72 hours. Oxygen was bubbled through one of the two batches for 10 minutes every 12 hours, while nitrogen was bubbled for 15 minutes through the second batch after it has been evacuated from air.

Cells of the two batches were collected separately, weighed and checked for radioactivity as described before. In each case cells were saponified with 10 percent methanolic sodium hydroxide for 5 hours.

Methanol was removed and the aqueous layer was exhaustively extracted with ether: chloroform (4:1, v/v). Results presented in table 4 show that whereas under anaerobic conditions the cell mass was 4 times of the aerobically grown cells, the incorporation of mevalonic acid into cellular lipids was approximately 50 percent less. It can also be seen that cellular radioactivity was quantitatively recovered in the non-saponifiable fraction which is in agreement with previous findings of Durr and Shwayri (13).

2. The ether: Chloroform extracts of aerobically grown cells (19×10^4 counts/minute) and anaerobically grown cells (316,500 counts/minute) were flash evaporated to dryness, resuspended in 3 ml of petroleum ether and applied to a deactivated alumina column (15 x 2 cm). The pattern of elution is shown in figure 7. This indicates a shift of the non-saponifiable lipids into more polar fractions in the presence of molecular oxygen.

B. Determination of Squalene and Sterols

The presence of squalene and sterols has not been shown in L. casei (18) we therefore attempted to investigate whether this was also true of L. plantarum.

One kgm of wet radioactive cells grown as described previously, was saponified and extracted with ether: chloroform (4:1, v/v). The ether: chloroform extract was flash evaporated. 5 ml of ether were added to the residue and the insoluble residue was centrifuged and saved. The supernatant solution contained (516,600 counts/minute). An aliquot of this fraction (32×10^4 counts/minute) was mixed with

TABLE 4

EFFECT OF OXYGEN ON THE GROWTH OF L. PLANTARUM
AND THE METABOLISM OF MEVALONIC ACID

Condition of Growth	Cellular mass (gms)	Cellular radio-activity counts/minute	Total Radio activity in non-saponifiable lipids counts/minute	Relative cellular radio-activity counts/minute/gm
Anaerobic	100	308,000	316,500	3,080
Aerobic	25	165,000	190,000	6,600

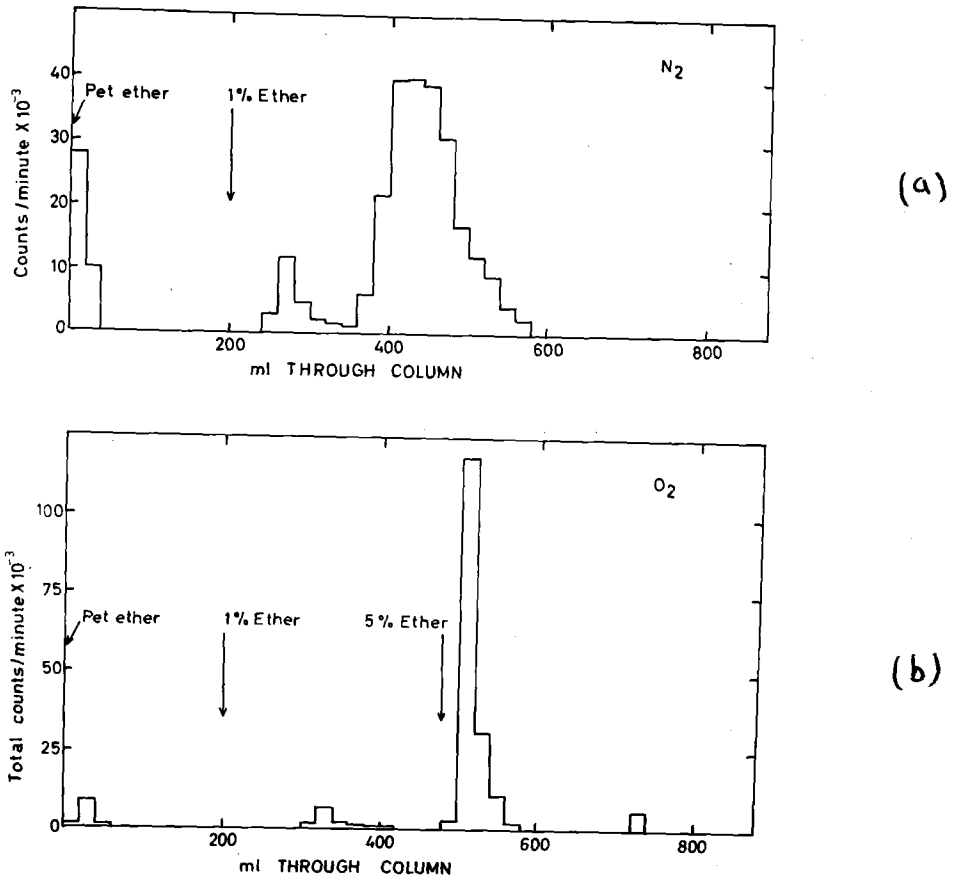


FIGURE 7. ELUTION PROFILES OF NON-SAPONIFIABLE LIPID EXTRACT OBTAINED FROM (a) CELL GROWN ANAEROBICALLY (b) CELLS GROWN AEROBICALLY ON DEACTIVATED ALUMINA COLUMN

0.5 ml of authentic squalene and applied to a deactivated alumina column (15 x 2 cm), according to Salokangas et al. (25). 20 ml fractions were collected using the solvents indicated in figure 8.

It can be seen that the n-heptane fraction contained a single peak (17,560 counts/minute) while the 5 percent ether fraction was resolved into at least two peaks (15,360 and 192,500 counts/minute) respectively.

1. n-heptane fraction (17,560 counts/minute) was evaporated under a stream of nitrogen. A yellow residue was obtained. The residue was suspended in 3 ml acetone and dry HCl gas was passed according to Heilborn et al. (26) through the tube containing the sample, which was kept at -15° by a dry carbon dioxide water bath. Heavy precipitate was obtained within 5 minutes. The reaction was continued for half an hour. The tube containing the precipitate was kept at room temperature to remove excess hydrochloric acid fumes. The precipitate was collected by centrifugation at 1000 x g, washed 4 times with cold acetone and 3 times with cold ether. The sample was kept under vacuum for 24 hours to dry, and subsequently an aliquot was counted. Squalene hexachloride contained no radioactivity.

2. The other fractions of this column were flash evaporated separately, each residue was dissolved in two ml ether. 0.2 ml of each solution was taken and dried under a stream of nitrogen. 5 ml of alcoholic potassium hydroxide was added to each tube. The tubes were stoppered, shaken well and incubated at $37-40^{\circ}$ for 55 minutes.

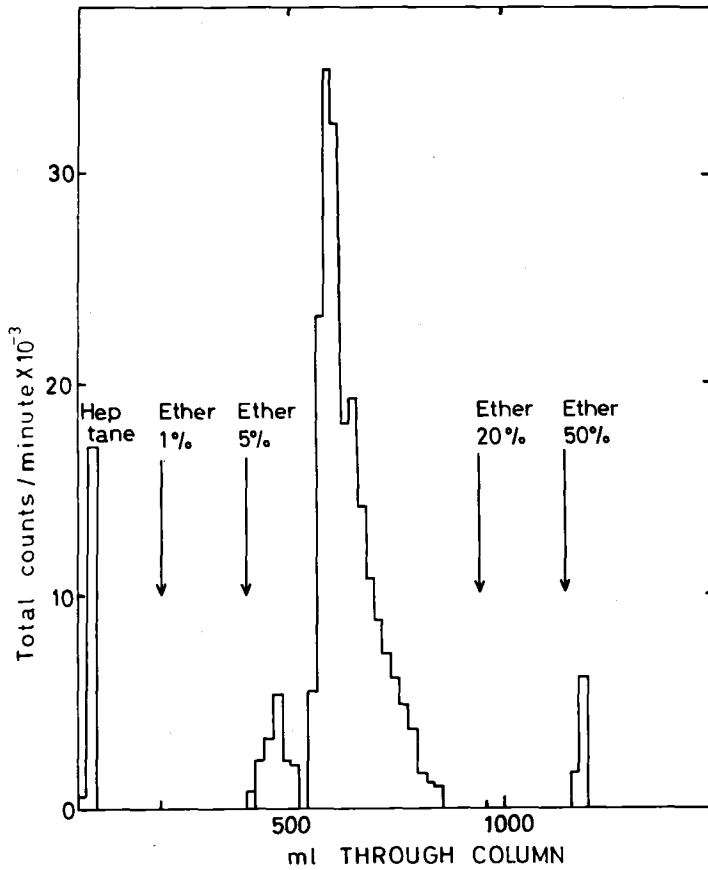


FIGURE 8. FRACTIONATION OF THE NON-SAPONIFIABLE
LIPIDS OF L. PLANTARUM
ON DEACTIVATED ALUMINA COLUMN

After cooling to room temperature, 10 ml of petroleum ether was added and mixed well with the contents of each tube. 5 ml of water were added to each tube and were shaken vigorously for 1 minute. 3 ml of petroleum ether layer was taken from each tube. Petroleum ether was evaporated under a stream of nitrogen and Liebermann-Burchard reagent was added for color development. A cholesterol standard (0.4 mg/ml) was similarly treated. The color developed was read at 625 m μ . The standard gave a dark green color with a reading of 0.75 mg of cholesterol while one of the fractions (15,350 counts/minute) eluted with 5 percent ether gave an olive-green color with a reading of 0.2. The ether heptane fractions were negative (27).

On the other hand, fractions which were not purified (80 percent alcohol, and (I₂) (figure 3) were fractionated further by the use of organic solvents. These fractions gave Liebermann-Burchard positive tests.

IV. PREPARATION OF AN EXTRACT OF L. PLANTARUM

Several techniques were adopted for the preparation of a cell free extract: trituration, sonnication, homogenization, and lysis with lysozyme. The protein content of the extract obtained by these techniques was very low (1-2 mg/ml) while a higher protein content (4-8 mg/ml) was obtained when an extract was prepared by the use of a Wabash hydraulic press.

Cells grown in 4 liters of semisynthetic medium of Skegg et al. for 20-24 hours were harvested, washed with distilled water and weighed.

8-12 gms of wet cells were obtained, suspended in 10 ml of 0.1 M citrate buffer pH 6.3 and pressed 3 times at 6000 lbs per square inch by the Wabash hydraulic press model 12-10 S. The pressed cells were centrifuged at 1000 x g for 10 minutes. The supernatant solution was collected and the residue was resuspended in 20 ml buffer. The residue was subjected to the same treatment twice. The volume of the extract was equal to the volume of the added buffer, with a protein content of 4-8 mgm/ml. The extract was dialyzed against 4 liters of the same buffer, for 3 hours. Incubation with mevalolactone-1-C¹⁴ (specific activity 4.5×10^5 counts/umole) was run for 3 hours. The incubation reaction was stopped by injecting 0.3 ml of 3N sulfuric acid into the flask. The liberated carbon dioxide was trapped with 1 ml of 1.N sodium hydroxide and checked for radioactivity. (Table V) shows that ATP as well as glucose were necessary for the decarboxylation of mevalolactone-1-C¹⁴. Furthermore (Table V) shows that the decarboxylation is inhibited by added NAD and NADP. It may be noted here that we have found that L. plantarum has an active pentose cycle.

TABLE 5

INCORPORATION OF MEVALOLACTONE-1-C¹⁴ BY A CELL
FREE EXTRACT OF L. PLANTARUM

The complete reaction mixture contained 25 mgs of protein (Crude extract) 6 umoles of glucose, 1 umole MgSO₄, 1.6 umoles NAD, 1.3 umole NADP, 1.6 umole ATP, 1 umole of DL-mevalolactone-1-C¹⁴ (4.5 x 10⁵ counts/minute) and 500 umoles citrate buffer pH 6.3 in a final volume of 5.7 ml.

Incubation was for 3 hours at 37°. The crude extract (30 ml) was dialyzed for 3 hours against 4 liters of the 0.1 M Citrate buffer pH 6.3.

<u>Reaction Mixture</u>	<u>Carbon Dioxide</u> <u>Total counts/minute</u>
Complete system	228
NAD and NADP omitted	1038
ATP omitted	216
Glucose omitted	360
NAD, NADP and ATP omitted	240

DISCUSSION

Studies on the stoichiometry of mevalonic acid metabolism by L. plantarum showed that for every mole of labelled CO_2 liberated from mevalolactone-1- C^{14} one mole of radioactive carbon was incorporated from mevalolactone-2- C^{14} into non-saponifiable lipids (13). Presumably, mevalonic acid in these microorganisms is converted into the biologically active isoprene unit as in the case of yeast, staphylococci, higher plants, and animal tissues (10, 19, 28), to yield a series of terpenes and sterols.

The non-saponifiable lipids of L. plantarum, derived from mevalonic acid, were extracted, fractionated, and purified as described under "Experiments and Results". Some of these fractions gave a positive Liebermann-Burchard test which suggests the presence of sterols. Due to the fact that some sterols do not give a positive Liebermann-Burchard test (29), one cannot draw conclusive evidence for the absence or presence of sterols in the other fractions. Hence more vigorous chemical analysis was necessary.

Squalene, a central intermediate in the metabolism of mevalonic acid by fungi, higher plants as Ocimum basilicum (30), Salvia officinalis (31), and animal sterols, has never been isolated from bacteria (32). Although some of the radioactive products of mevalonic acid isolated from L. plantarum migrated like squalene on a deactivated alumina column, these radioactive products were not identical with squalene. This was shown by adding authentic squalene to the radioactive fraction, and crystallizing it as the hexachloride. No radioactivity was associated

with the crystallized squalene derivative.

We have isolated one component (I_{14}) from the non-saponifiable lipids of L. plantarum. The following lines of evidence suggest that (I_{14}) is a pure compound: a) It has a sharp melting point 20-21°, b) It gave a single spot with five different solvents, c) Presence of some sharp bands in the infra-red spectrum. This compound has been identified as a saturated monoester with an empirical formula $C_{26}H_{51}O_4$ and a molecular weight of 426.

Various chemical analyses were performed on (I_{14}) to determine its functional groups. In addition (I_{14}) was also scanned in the infra-red region of the spectrum. The presence of a diffused band, 3445 cm^{-1} , in the infra-red spectrum (figure 6.) suggests an associated (H_2O). That this association is in an ester form is indicated by the strong, sharp peak at 1750 cm^{-1} , which is typical of saturated esters (33). Colorimetric determination by the hydroxamate method, and the results of the saponification experiments indicated that the compound is monoester.

The extraction of the lipids derived from mevalonic acid in the absence of alkalies and acids allowed for a minimum alteration of the form in which these compounds exist. Thus (I_{14}) a monoester is among the few metabolites of mevalonic acid to be isolated in the ester form. Trichothecin, a metabolic product of the mould Trichothecium roseum arising from mevalonic acid, is also a monoester of cis-crotonic acid (34).

The C:H ratio, an iodine value of zero, and lack of absorption in the ultra-violet region of the spectrum show that (I_{14}) is a saturated hydrocarbon. This is further supported by the infra-red spectrum. The prominent sharp bands 2860, and 2930 cm^{-1} and a lesser one 1470 cm^{-1} indicate that the major part of the compound consists of a saturated aliphatic ($-\text{CH}_2$) paraffin (33). However, several considerations lead us to believe that (I_{14}) is not completely aliphatic, and that it has a cyclohexane moiety.

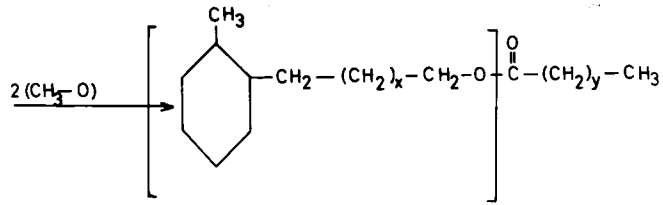
Elementary analysis shows that the C:H ratio is between $n:2n+2$ (saturated and aliphatic) and $n:2n$ (alicyclic) suggesting that the compound could not be completely acyclic or aliphatic. The weak diffuse bands in the infra-red spectrum 970-1050 cm^{-1} are typical of cyclohexane derivatives. The presence of alicyclic compounds in the lipids of lactobacilli has been known since lactobacillic acid ($\text{C}_{19}\text{H}_{36}\text{O}_2$) was shown to contain a cyclopropane ring (14). On the other hand, vitamin A and related compounds contain a derivative of cyclohexane (35).

The remaining oxygen atoms could not be in the form of a free (OH) since the (OH) number was zero. The presence of epoxide systems is excluded since this will be inconsistent with C:H ratio. The presence of diffused infra-red bands, 1100-1175 and 1200-1250 cm^{-1} suggests presence of ether groups. Hence these oxygen atoms are probably in the form of methoxy groups. This is confirmed by the fact that the non-saponifiable fraction gave a positive Zeisel test. On the other hand, the saponifiable part was negative. Although a

Zeisel determination does not qualitatively differentiate between methoxy and ethoxy groups, the former is more likely to be present, since ethoxy groups are not found in biologically occurring compounds. Whether these methoxy groups arise from mevalonic acid or from one carbon fragments remains to be established. The contribution of one-carbon donors to the synthesis of polyprenes is known. A case in point is ergosterol where C-28 is derived from methionine (36).

Chemical analysis of (I_{14}) also indicated the presence of 2 ($C-CH_3$) groups. One of these would be the terminal methyl group of the acid moiety of the ester. The other one is probably part of the terpenic alcohol. The presence of only 2 ($C-CH_3$) groups in (I_{14}) indicate that the polymerization of the isoprene unit is accompanied by oxidative demethylation since for every isoprene unit there is one ($C-CH_3$) group, which is not found in (I_{14}). The transformations of terpenes are frequently accompanied by a migration, an oxidation, or a removal of these methyl groups. Thus lanosterol, for example loses three methyl groups during its conversion to cholesterol. This is equivalent to the loss of one methyl group for every 2 isoprene units.

Obviously, further information is necessary to elucidate the structure of (I_{14}). However, the integration of the available information into a hypothetical structure for (I_{14}) may aid in delineating the approach to the ultimate solution of its structure. Furthermore, any proposed structure will have to take into consideration the mechanism of polymerization of the isoprene units. The formation of (I_{14}) can be visualized according to the scheme in figure 9. Isoprene



I 14 $x+y=13$

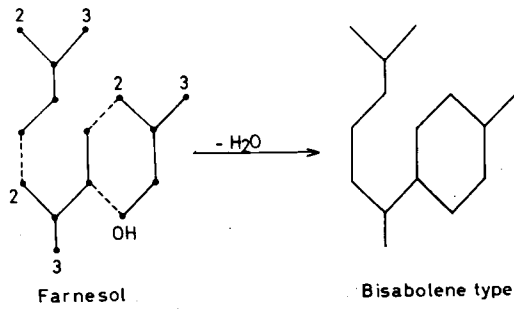
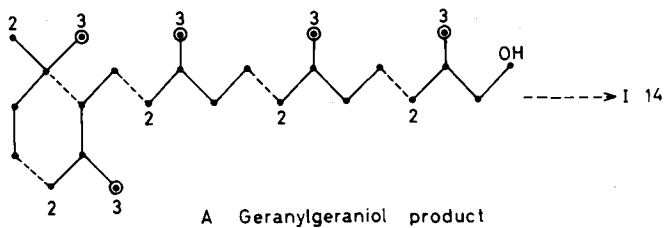


FIGURE 9. A POSSIBLE STRUCTURE FOR I₁₄ AND ITS BIOGENETIC SCHEME

units polymerize via carotenoid rather than a squalene pathway. The former yields geranylgeranio derivatives (C_{20}), whereas, the latter yields farnesol (C_{15}). The absence of farnesol or squalene among the non-saponifiable lipids of L. plantarum indicates that the use of the farnesol pathway is unlikely. In addition, the extension of farnesol to yield longer intermediates is not known. Furthermore, farnesol intermediates yield a bisabolene (C_{15}) type of compound (figure 9.) in which the alcohol function is lost during cyclization, whereas the geranylgeraniol intermediates can cyclisize without losing the hydroxyl group (37). The possible presence of a maximum of one ($C-CH_3$) in the alcohol moiety of (I_{14}) indicates that oxidative demethylation occurs during the formation of (I_{14}); one methyl group will be lost for each isoprene unit. As mentioned previously the loss of methyl groups, leaving behind an incomplete terpene polymer, is a common finding among biologically occurring polyprenes. It is interesting to point out that this hypothetical scheme for the biogenesis of (I_{14}) can be tested by using mevalonic acid labelled in both the ($C-CH_3$) group, and ($C-2$) which represents the other methyl group of the isoprene unit. It is well known that the ($C-CH_3$) is found as the branched methyl group of squalene and other terpenes, whereas ($C-2$) is in the backbone of the molecule. Since the branched methyl groups of terpenes are lost as CO_2 , this should give rise to labelled CO_2 in amounts equivalent to the label appearing in (I_{14}). Even when these two groups are present in a terminal isopropylidene

or a gem-dimethyl group, they retain their stereochemical individuality and thus, are acted upon differentially. For example, in Sayasapogenol, a cyclic triterpene, the gem groups, (C-23), (CH₃), and C-24 (CH₂OH) arise from (C-CH₃) and C-2 of mevalonic acid respectively (36).

We have also explored the possibility of using whole cells and cell free extract to study the mechanism of mevalonic acid metabolism. In view of the fact that some of the non-saponifiable lipids were oxygenated products we attempted to investigate whether molecular oxygen had any effect on the elution pattern of the products derived from mevalonic acid. The findings that anaerobiosis partially inhibited the synthesis of non-saponifiable lipids, and the shift of radioactive non-saponifiable lipids under aerobiosis towards the more polar products, suggest that oxygen is needed for the transformation of the various metabolites. Studies with cell-free extracts indicated that the metabolism of mevalonic acid is dependent upon glucose and ATP, in analogy to other biological systems. However, the methods available for preparing cell-free extracts from lactobacilli were not adequately effective in breaking the cell, nor were they reproducible. Hence a more detailed study of the mechanism of mevalonic acid incorporation into the various non-saponifiable lipids will have to await better methods for preparing cell-free extracts.

SUMMARY

This research program was designed to isolate and identify intermediates of mevalonic acid metabolism by L. plantarum, to study the pattern of these intermediates on column chromatography, and to investigate the effect of different conditions on the growth and metabolism of the organism. The accumulated products of mevalonic acid in these cells were extracted, fractionated, and purified by organic solvent precipitation, and chromatography. (I₁₄), one of the isolated intermediates was purified and identified as a mono-ester with an empirical formula of C₂₆H₅₁O₄. Lines of evidence for the presence of a cyclohexane ring, two methoxy, and two side-chain methyl groups in its structure were given.

Under anaerobic conditions the cell mass was 4 times that of the aerobically grown cells while the incorporation of mevalonic acid into cellular lipids was approximately 50 percent less. It was shown that cellular radioactivity was quantitatively recovered in the non-saponifiable fractions. Squalene was not present among these fractions.

Preliminary studies on cell free extracts indicate that the metabolism of mevalonic acid requires ATP and glucose.

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