MICROBIOLOGICAL TRANSFORMATIONS OF TERPENES

A Thesis

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By

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CHAPTER - I

I N T R O D U C T I O N

INTRODUCTION

A review of literature would indicate that although extensive investigations on the microbiological conversions of steroids have been carried out, relatively little work has been done on the microbiological degradation of terpenoid compounds. Only during the last five years some interest has been apparent in this field.

Though the systematic investigation on the microbiological transformation of terpenoid compounds started from 1959, the work on this line dates back to 1929, when Molinari (1929) prepared citronellic acid (2) from citronellal (1) using Acetobacter xylinum.

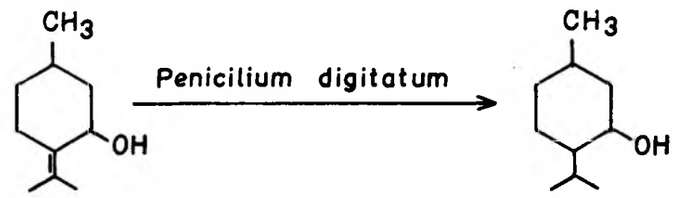
(1) (2)

Neuberg and Pieser (1949), while studying the phytochemical reductions of terpenoid compounds with baker's yeast, observed that 2,3,diketo-camphane (camphor quinone) (3) is reduced in 63% yield to 3-hydroxycamphor (4).

(3) (4)

They also observed that with the same baker's yeast dextrocamphorquinone gives dextrorotatory hydroxy camphor while the racemic d,l,camphorquinone gives only the corresponding levorotatory hydroxycamphor.

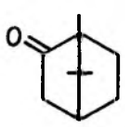
Babička and co-workers (1955) carried out the fermentation of citronellol, pulegol (5) or isopulegol using Penicilium digitatum, and obtained menthol as the conversion product. The process consisted in propagating the culture of Penicillium digitatum for 48 hours at 22° on 1.5% brewers wort. Citronellol, pulegol (5) or isopulegol was added to a final concentration of 2% and the culture was allowed to grow for 28 days at 22°. Menthol (6) was then separated in 93% yield by steam distillation, freezing and centrifugation. The residue containing the unreacted substrate was sterilized and used in the next batch.



PULEGOL (5) MENTHOL (6)

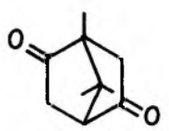
The first systematic studies on microbiological transformation of terpenoid compounds, such as camphor, were initiated by Corey and Gunsalus (Bradshaw et al 1959) by a Pseudomonad strain C_1 (also called P) isolated from sewage

sludge by enrichment culture techniques using (+) camphor (7) as a carbon source. Extraction of the broth at the end of the logarithmic phase of growth separation of the extractives into neutral and acidic fractions on camphor, and chromatography of the neutral fraction yielded three products: 2,5-diketocamphane (8), and two hydroxy ketones which proved to be 5-exo (9) and 5-endo (10) hydroxy camphors respectively.



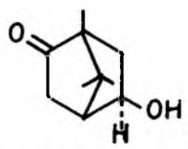
CAMPHOR

(7)



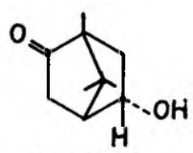
DIKETOCAMPHANE

(8)



5-EXO

(9)

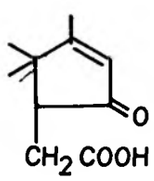


5-ENDO

(10)

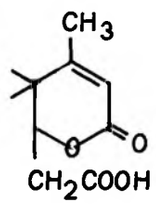
Hydroxy camphor

The acidic fraction afforded a keto acid, $C_{10}H_{14}O_3$, 3,4,4'-trimethyl-5-carboxy methyl Δ^2 cyclopentenone (11). Complete oxidation of thisacid by the resting bacterial cells was inhibited by 2,2'-bipyridine with the accumulation of a new lactonic acid (12) intermediate.



3,4,4',TRIMETHYL, 5,CARBOXYMETHYL CYCLOPENTENONE

(11)

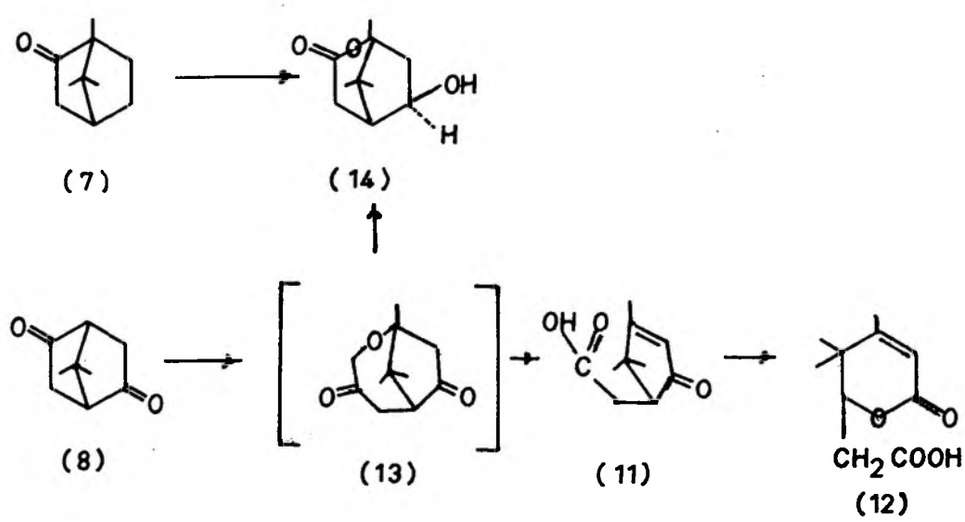


LACTONIC ACID

(12)

The pathway of degradation of camphor by this organism was partially formulated as (+) camphor ⟶ 5-hydroxy camphor ⟶ (8) ⟶ (11) ⟶ (12).

The ring cleavage reactions of the carbocylic rings of camphor were later shown to take place with the cell free extracts (Conrad et al 1961a) which catalyse the conversion of camphor (7) to 1,2,camphyllide (14) and 2,5,diketo camphane (8) to 3,4,4'-trimethyl-5-carboxymethyl Δ^2 cyclopentenone (11). The formation of compound (11) from (8) appeared to proceed via the 1,2,lactone (13) which presumably underwent hydrolysis and dehydration to yield the cyclopentenone(8).



Both the ring cleavage reactions required added NADH and consumed one mole of O_2 . An additional co-factor requirement was shown by dialysis or carbon treatment that inactivated the enzymes which could be reactivated with boiled extract. Dialysis in presence of bipyridine also inactivated the enzymes (and) Fe^{++} and boiled extract restored the activity. Cysteine and to some extent glutathione stimulated the activity.

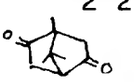
Hedegaard et al (1961) also found that two strains of pseudomonas C_1 and C_5 oxidised camphor (7) but differ in their ability to grow on this compound. The levels of induced enzymes and the nature of the products isolated as a function of time with growing and resting cells indicated more than one pathway for the degradation of camphor. They found that glucose-grown cells were inhibited both by camphor (7) and 1,2,camphyllide (14) even though camphyllide appeared to be a neutral metabolite of camphor. Intact cells of C_1 did not accumulate compound (14) but the cell free extracts did.

In continuation of this work Gunsalus and his collaborators (Conrad et al 1961b) obtained from the Pseudomonad C_1 strain a cell free extract which when supplemented with NADH and in presence of oxygen converted diketocamphane (8) to the cyclopentenone (11) in addition to the camphyllide (14) presumably through the intermediate (13). This extract also contained a NAD-linked dehydrogenase which converted the camphyllide (14) to the cyclopentenone (11) presumably through (13). A purified enzyme preparation also converted compound (8) to compound (11) stoichiometrically. It was shown that the lactonization reaction (8→13) required FMN, Fe^{++} and two enzyme fractions, E_1 (an FMN reductase) which supplies the electrons and E_2 (a lactonizing enzyme) which appears to undergo oxidation and reduction according to the following sequence.

[I] $NADH + H^+ + FMN \xrightarrow{E_1} NAD^+ + FMNH_2$

[II] $FMNH_2 + E_2 \text{ --- } 2Fe^{+++} \longrightarrow FMN + E_2 - 2Fe^{++}$

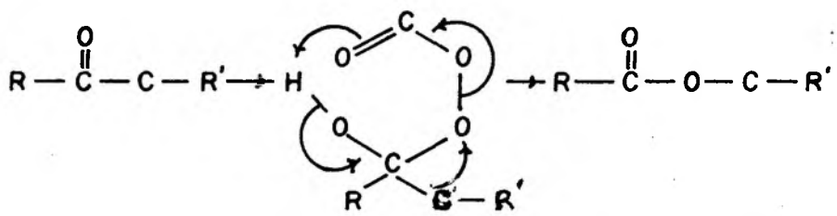
[IIa] $FMNH_2 + O_2 \longrightarrow FMN + H_2O_2$

[III] $E_2 - 2Fe^{++} + O_2 +$  $\longrightarrow E_2\text{-}2Fe^{+++} +$  $+ H_2O$

The E_1-dependent stage was insensitive to bipyridine. Neither was E_2 inhibited by bipyridine. However the total reaction was inhibited. It was found that after reduction with the FMN-reductase system (I and II) E_2 reacted with bipyridine and revealed 2 moles of Fe^{++}.

The lactonizing enzyme was purified 50 fold (Conrad et al 1962) and was shown to behave as a single component in ultracentrifugation and starch gel electrophoretic studies. The preparation showed a typical hemoprotein spectrum with a Soret-band at 410 mμ and can be reduced by $FMNH_2$ or dithionite, with the formation of α- and β-bands at 551 and 522 respectively and an intensification of the Soret band with a shift in the max. to 415 mμ. It was further shown that the home group is not dissociated from the protein by acid-acetone nor by silver salt treatment. Flavin is not present in this fraction as indicated by difference spectra.

The mechanism of lactonization corresponds to Bacyer-Villager oxidation of ketones to esters (Meinwald and Frauenglass, 1960) and presumably follows the following pathway involving the attack of a peroxide on the electrophillic carboxyl carbon.



In the enzyme reaction the nature of the attacking peroxide has not been elucidated so far.

Using enrichment culture techniques, Werner Seubert (1960) isolated a new species of the genus Pseudomanas (p) (Pseu.citronellolis) which oxidises citronellal and farnesal, as well as a number of intermediate isoprenoid degradation products. Extending the studies to the metabolism of acetate in alcohol-dried powder of the cells and using labelled carbon he also demonstrated that the substrate, citronellal, a likely intermediate in isoprenoid degradation, is partially converted to an ether-soluble fluorescent pigment and a number of amino acids. The structure of the fluorescent pigment has not been elucidated.

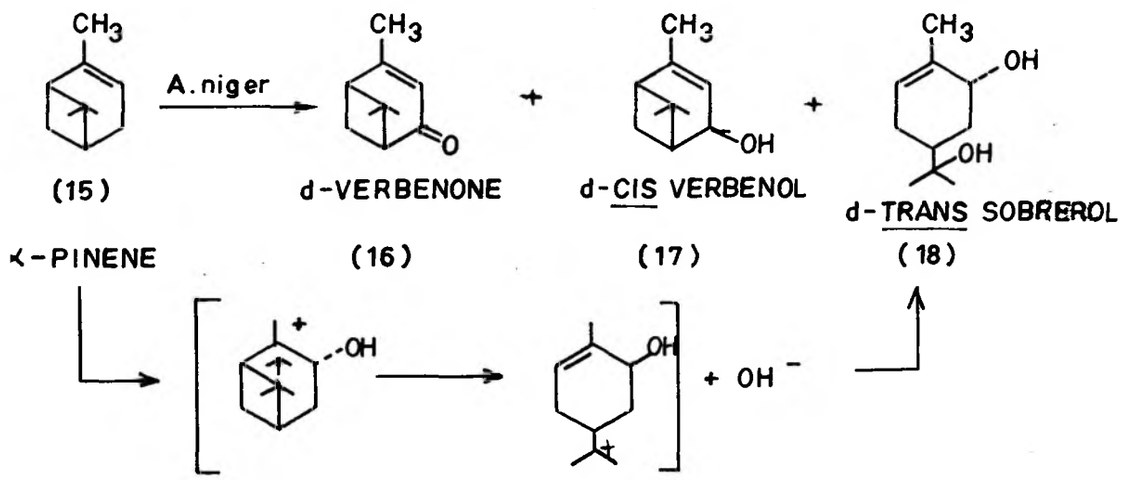
It is interesting to note that agarwood oil is obtained from the plant, Aquillaria agallocha Roxb, only when it is infected by fungal growth. Healthy plants do not contain any appreciable quantity of the oil. Agarwood which is odoriferous consists of irregular patches of dark streaks highly impregnated with an oleoresin formed due to fungal infection and is found in the interior of

comparatively old and mature trees. (Bose, 1938; Sadgopal and Varma, 1952).

From the infected agarwood a number of oxygenated compounds have been isolated by Bhattacharyya and co-workers (1959, 1963) which include, agarol, dihydroagarofuran, β-agarofuran etc. The non-infected agarwood contains mostly hydrocarbons.

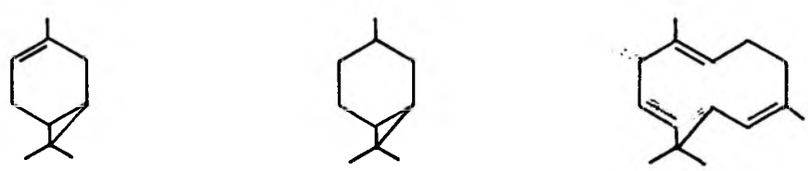
A screening programme was started in this laboratory following this lead to develop micro-organisms capable of bringing about chemical transformation of terpenoid compounds. Bhattacharyya et al (1960) published a preliminary note on the microbiological transformation of α-pinene (15). It was shown that three oxidation products were formed when α-pinene was incubated with pellets of an A.niger strain (NCIM 612).

Continuing their studies Prema and Bhattacharyya (1962a) found that the strain NCIM 612 of A.niger showed a marked ability to metabolise α-pinene. They also worked out optimum conditions of time, temperature and substrate concentration for maximal conversion. Three major oxidation products from α-pinene were identified, viz. a ketone, $C_{10}H_{14}O$, identified as d-verbenone (16); an alcohol $C_{10}H_{16}O$, as d, cis-verbenol (17) and a crystalline diol, $C_{10}H_{18}O_2$ characterized as a d-trans-sobrerol (18).



The formation of verbenol (17) and verbenone (16) was explained by oxygenation at the allylic position, while trans-sobrerol was probably formed by the attack of the electrophilic hydroxyl [OH^+] at the double bond, followed by a rearrangement and subsequent addition of OH^- to the carbonium ion.

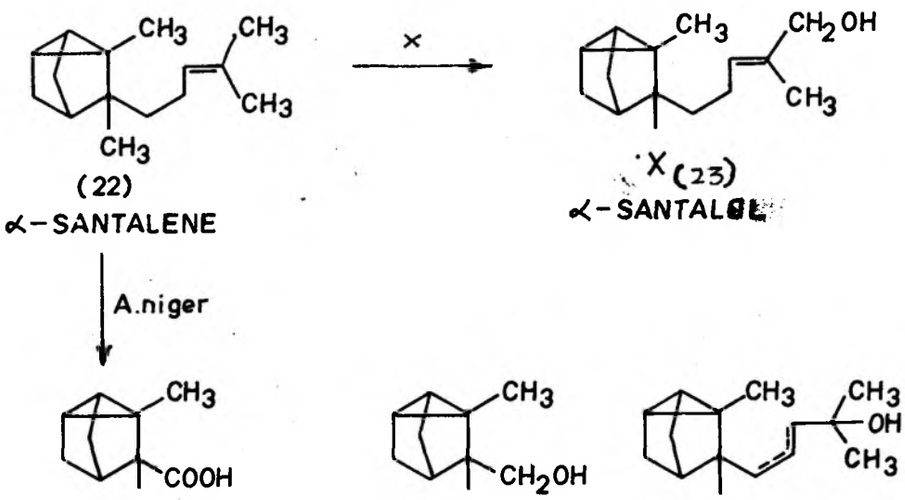
While studying the transformations of other hydrocarbons the same authors (1962b) noticed that Δ^3-carene (19), carane (20) and humulene (21) are resistant towards the microbiological action of the experimental strain of A.niger.



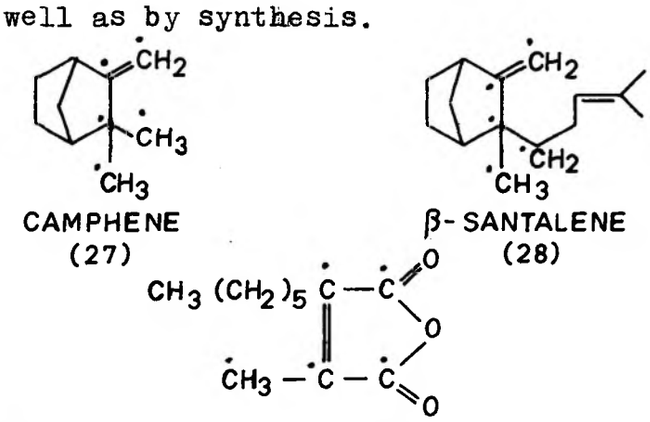
Δ^3-CARENE (19) CARANE (20) HUMULENE (21)

With a view to obtaining α-santalol (23) from α-santalene (22) by using the same strain of A.niger (612),

Prema and Bhattacharyya (1962b) observed that α-santalene (22) was degraded mainly to tere-santalic acid (24), tere-santalol (25) and a small quantity of a tert C_{15} alcohol (26).



After fermentation of camphene (27) and β-santalene (28) with A.niger 612, Prema (1962) obtained a dicarboxylic acid, which could only be isolated as an anhydride, $C_{11}H_{16}O_3$ (29), even under the mildest of conditions. The structure of the anhydride was established as 2-nonene-2,3-dicarboxylic acid anhydride (29) through physico-chemical studies as well as by synthesis.



2-NONENE-2,3-DICARBOXYLIC ACID ANHYDRIDE

Assuming that the five carbon dicarboxylic acid anhydride portion isactually derived from these terpenoid substrates (27 & 28), Prema (1962) postulated a mechanism for its formation based on the type of reaction involving a bond shift which leads to sobrerol formation from α-pinene oxide. She based these speculations on the finding that controls run without the terpene hydrocarbon did not seem to produce the anhydride.

The above finding, however, could not be regarded as the absolute proof for the terpenoid origin of the anhydride. In all the fungal fermentations, carbon sources other than these terpenes were also present in the medium. Therefore, there can be three possibilities regarding the origin of the anhydride. (a) It can be a true transformation product of the terpenoid substrate. (b) It could be entirely derived from carbon sources of non-terpenoid origin and (c) It can be partly derived from the terpene as well as partly from others substrates present. It was not clear from Prema's work (1962) which one of the three possibilities underlies the origin of the anhydride.

At the preliminary stages of the present work it was observed that the same anhydride was obtained when longifolene (30) was incubated with A.niger under the conditions developed by Prema (1962). The current investigation started with a more detailed study on the

structure and the origin of 2-nonene-2,3,dicarboxylic acid anhydride (29).

Object and Scope of the present investigation

The meagre data in the literature on the subject of microbiological transformation of terpenes indicate that this is a field in which much work remains to be done. Preliminary results obtained in thislaboratory are encouraging. From the data reviewed in the previous section it will be seen that micro-organisms show a remarkable versatility in metabolizing terpenoids of different types of structure and carry out a variety of reactions such as hydroxylation, oxidation and carbon-carbon bond cleavage and other transformations. In order to assess more realistically the limits and scope of these microbial transformations,it was considered necessary to extend the work further to a number of terpenoid compounds and to other types of micro-organisms besides the fungi.

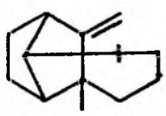
These investigations were,furthermore, undertaken with two main objectives. (1) To get some understanding about pathways of biosynthesis and biological conversions of terpenoid compounds in nature. (2) To explore the possibility of developing new fermentations yielding products of interest to the perfumery industry.

Present Work

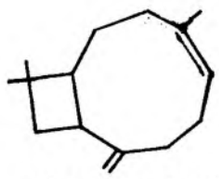
(1) Terpene transformations by fungi

In the present work it has been shown that 2-nonene, 2,3-dicarboxylic acid anhydride can be obtained by the incubation of longifolene (30) with A.niger and that the rate of accumulation of anhydride follows closely that of the disappearance of longifolene. Further degradative and synthetic studies in confirmation of the structure of the anhydride are presented.

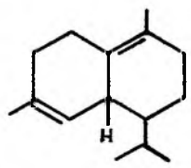
It has also been found that fermentation of two other hydrocarbons, caryophyllene (31) and δ-cadinene (32) also results in the formation of the same anhydride (2-nonene-2,3-dicarboxylic acid anhydride) (29). Furthermore, it was possible to detect small amounts of the anhydride by paper chromatography in control experiments run without the terpenoid hydrocarbons.



LONGIFOLENE
(30)



CARYOPHYLLENE
(31)



δ-CADINENE
(32)

By the use of glucose-U-C^{14}, and unlabelled longifolene it has been possible to show that the anhydride can entirely be derived from non-terpenoid substrate carbons. The origin of the anhydride appears, therefore to lie in a possible condensation of octanoate or its equivalent with pyruvate.

(2) Fermentation of β-pinene

Fermentation of β-pinene (52) by A.niger has been presented in the next chapter. The isolation of a monohydroxy alcohol from the fermentation mixture and its characterization as β-terpineol (53) is discussed.

(3) Terpene transformations by bacteria

The last chapters of this thesis deal with the isolation and characterization of a soil pseudomonad capable of growing on limonene (55) as the sole source of carbon. Preliminary studies on the organism with respect to its growth and nutritional requirements are reported.

Fermentation of limonene by this bacterium resulted in the formation of a large number of neutral and acidic transformation products in small quantities. These products were separated into various individual components by using techniques such as solvent-solvent distribution, chromatography as well as vapour-liquid partition chromatography.

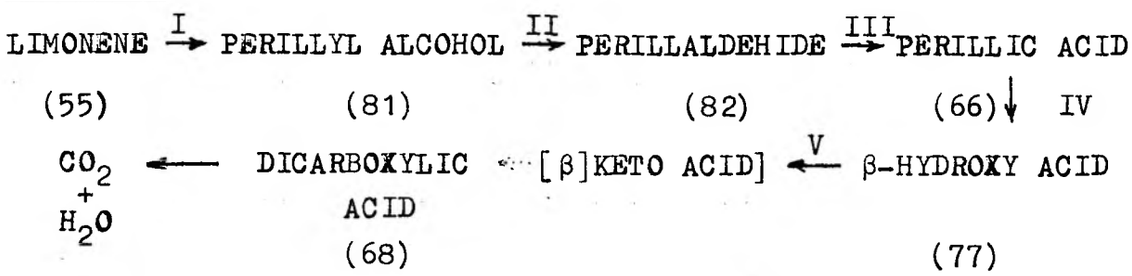
Six of the neutral compounds were identified as carvone (57), dihydrocarvone (56), carveol (58), p-menth-8-ene, 1-ol-2-one (60), p-menth-8-ene-1,2,diol (62) and p-menth-6-ene, 2,9,diol. (64). From the acidic fractions the following were isolated and identified : perillic acid (66), 2-hydroxy-p-menth-8-ene-7-oic acid (77), β-isopropenyl-pimelic acid (68) and 2,9-dihydroxy,- p-menth-6-ene-7-oic acid (79). In addition to the above compounds several

others could be isolated from the fermentation mixture. The compounds were available in insufficient quantities for complete characterizations.

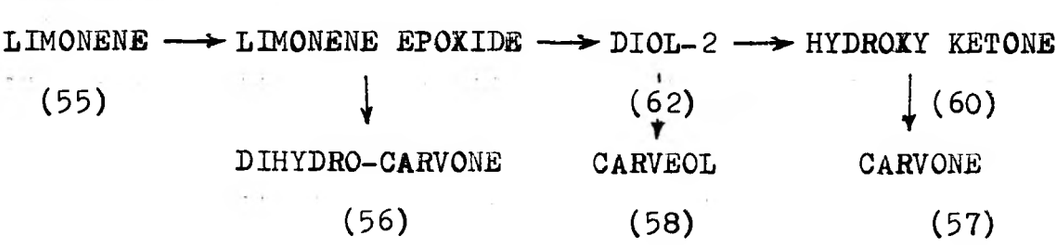
(4) Enzymatic studies

In the final chapter enzymatic studies on the mechanism of limonene degradation have been reported. Growth and adaptive enzyme studies indicated that none of the neutral compounds (56,57,58,60 and 62) is on the direct pathway of oxidation of limonene. The acidic compounds (66,68 and 77) are all oxidised by limonene-grown cells. Limonene (55) added to cells grown on glucose is converted mainly to perillic acid (66), and in small amounts to perillyl alcohol (81). Perillic acid-grown cells accumulate β-isopropenyl pimelic acid (68). Based on these data the oxidation of limonene has been explained by two probable main pathways : (Pathways 1 and 2 : Chapter VI).

PATHWAY 1



PATHWAY 2



The unfractionated cell-free-sonicates from the limonene grown cells carry out the entire sequence of reactions I to V (Pathway 1, Chapter VI) in the presence of added co-factors such as NADPH, NAD, FAD, FMN and ATP, cysteine and Mg^{++} ions. It has been shown that the first reaction in pathway 1, the hydroxylation of limonene (35) in the presence of NADPH and oxygen, is carried out by the sediment (particulate) obtained on centrifugation of the cell free extract at 100,000 g. The supernatant was precipitated at 0.9 saturation with ammonium sulphate at 0^{o}. The precipitate obtained has been shown to convert perillyl alcohol (81) to perillic acid (66) with NAD, and to transform perillaldehyde (82) to perillic acid (66) in the presence of methylene blue, FAD and/or FMN. It also converts perillic acid (66) to the dicarboxylic acid, β-isopropenyl-pimelic acid (68), in the presence of ATP, CoASH and NAD. The enzymatic activities have been monitored throughout by thin layer chromatography and vapour liquid partition chromatography.

CHAPTER - II

MATERIALS AND METHODS

MATERIALS AND METHODS

The chemicals used in the media for the growth of Aspergillus niger and for the bacterium were of 'chemically pure' (CP) quality, and those used in the enzyme work were of analytical grade (A.R.). Longifolene, β-pinene, δ-cadinene and carvone were kindly supplied by Dr. S.C. Bhattacharyya, Head of the Essential Oils Division of this laboratory. A sample of perillic acid (synthetic) was obtained from Dr. Prof. Kergomard, France. Perillyl alcohol and perillaldehyde were synthesised from perillic acid. Zwischenferment was prepared according to the method of Jagannathan et al (1956). NAD, NADP, ATP, FAD, FMN, G-6-P, etc. were obtained from Sigma Chemicals Co. Glass distilled water was used in all manometric and enzyme work.

The fermentations were carried out either in rotary shake cultures at 220 r.p.m. or with stainless steel fermentors of 6 l capacity with stirring arrangement (720 r.p.m.). The fermentations were carried out at $28^{\circ} \pm 1^{\circ}$.

All melting and boiling points are uncorrected. Optical rotations were measured without solvents or in chloroform (unless otherwise specified) and were determined in a Hilger standard polarimeter using a 1 dm tube. The

infrared spectra were recorded on a Perkin Elmer Infracord model 137-B and E, spectrophotometer or on a Grubb-Parson--Double beam spectrophotometer either in smears (liquids) or in nujol (solids) unless otherwise stated. The ultraviolet measurements were made on Beckman model DU, spectrophotometer in 95% ethanol solutions. Gas chromatography was carried out on a Griffin-Tatlock model G, Mark III or on Perkin Elmer vapour aerograph analytical model (A 350 B) using hydrogen as the carrier gas. Thin-layer chromatography was carried out with 85% silica gel (Merck 200-400 mesh) in 15% plaster of Paris as the supporting medium. The plates were sprayed with concentrated sulphuric acid. Paper chromatograms were run on Whatman filter paper No.1. All n-m-r spectra were taken in a 20% solution in carbon tetrachloride with tetramethyl silane as the internal standard in a Varian Associates A-60, Spectrometer. Solvent extracts were finally washed with brine and dried over anhydrous sodium sulphate. Pet-ether refers to the fraction boiling between 40-60°. Neutral alumina, Brockmann (1955) grade II was employed throughout.

The following abbreviations have been used.

TLC : Thin layer chromatogram.

VPC : Vapour phase chromatogram.

I.R. : Infra-red.

n-m-r : Nuclear magnetic resonance.

UV : Ultraviolet.

ATP : Adenosine triphosphate.

NAD : Nicotinamide adenine dinucleotide.

NADP : Nicotinamide adenine dinucleotide phosphate.

NADPH : Reduced nicotinamide adenine dinucleotide phosphate.

G-6-P : Glucose-6-phosphate.

Z.F. : Zwischenferment.

FAD : Flavin-adenine dinucleotide.

FMN : Flavin mononucleotide.

CMP : Chloramphenicol.

MB : Methylene blue.

Tracer studies

C^{14}-uniformly labelled glucose was obtained from the Radio Chemical Centre, Amersham, England [CFB 35, Batch 8, D-glucose - C^{14} (U) Vacuum sealed, 1.22 mg; 0.5 mc].

Radioactive countings were monitored initially on 'Super Scaler', Tracer Lab., U.S.A. and finally estimated on the Tri-carb liquid scintillation spectrometer system, model 314, Ex-2-Packard Instruments Co. Inc., U.S.A., using the scintillation fluid consisting of :

Naphthalene	15 g
POP	1 g.
Dimethyl POPOP	50 mg
Methanol	25 ml
Ethylene glycol	5 ml

made up to 250 ml with methanol.

Electron Micrograph

Electron micrographs of the limonene grown bacterium were taken on an Electron microscope at the Indian Cancer Research Institute, Bombay, and the Indian Institute of Science, Bangalore.

[A] FUNGAL TRANSFORMATION

(1) Maintenance of culture and propagation of spores for inoculation of liquid medium.

Aspergillus niger (Strain, 612, National Collection of Industrial Micro-organisms, National Chemical Laboratory, Poona, India) spores were maintained on potato--dextrose-agar (PDA) slants according to Fred and Waksman (1928) having the following composition :

Potato	20 g
Dextrose (B.D.H.)	2 g
Agar (Algader, Denmark)	1.5 g
pH adjusted to	5.5

made up to 100 ml

Pealed potatoes (20 g) were sliced and cooked in 80 ml of water for 1 hr in the steamer. The mash was filtered and to the filtrate, after cooling, 2 g of glucose and 1.5 g of shreded agar were added; the medium was diluted to 100 ml by the addition of distilled water and steamed for 30 min. For the propagation of slants 8-10 ml of the

medium were taken in 25 ml Pyrex test tubes and autoclaved at 15 psi (121°) for 20 min. These slants were inoculated from the stock culture and incubated at 28° for 6 to 7 days.

(2) Medium for growth and for inoculum

Modified Czapeck-Dox medium (Prescott, 1959) used for the growth of A.niger had the following composition.

Glucose (Danpha)	40 g
Potassium chloride (Bengal Chemical)	0.5 g
Magnesium sulphate, anhydrous (B.D.H.)	0.25 g
Sodium nitrate (B.D.H.)	2.00 g
Potassium dihydrogen phosphate (Thomas Tyrer)	0.8 g
Dipotassium hydrogen phosphate (Thomas Tyrer)	0.2 g
Ferrous sulphate, $7H_2O$ (Danpha)	10 mg
Yeast extract (Oxoid)	0.5 g
Corn steep liquor (Anil Starch)	5.0 g

made up to 1 l.

The pH was adjusted to 4.6 to 4.8 either with phosphoric acid or with dipotassium hydrogen phosphate.

Sterilization :

The medium (4 l) was either transferred to a stainless steel fermentor, or aliquots of 100 ml of the growth medium were distributed in 500 ml Erlenmeyer flasks and autoclaved at 15 psi for 20 minutes, and inoculated with a spore suspension in sterile distilled water from 6 day-old potato dextrose-agar (PDA) slants.

Incubation :

The inoculated fermentors or flasks were incubated at $28^{\circ} \pm 1$. After a good growth was obtained (normally 24-30 hr), the substrate (longifolene, caryophyllene (δ-cadinene or β-pinene) was added aseptically in different concentrations depending upon the rate of oxidation, and the fermentation was allowed to continue for 24 hr with periodic sampling for analysis and microscopic examination. Any bacterial contamination was usually detected by the determination of the pH which would show a rise in case of bacterial contamination and by staining the broth smears by the standard gram-staining procedure.

Controls were run with the inoculum and without the terpenoid substrates as well as without inoculum and with terpenoid substrate.

(3) Extraction

For the estimation of the conversion products the following extraction procedure (chart 1) was adopted. The fermented medium was strained through muslin and the fungal pellets were extracted twice with acetone (2 volumes) and then with ether ($\sim$ 2 volumes). The pooled extracts were used to extract the aqueous layer (broth) which was subsequently extracted with fresh ether (1/10th of the total volume x 3). The solvent layers were pooled. After the ether extraction both the mycelium and the broth were extracted with n-butanol as described above.

EXTRACTION PROCEDURE

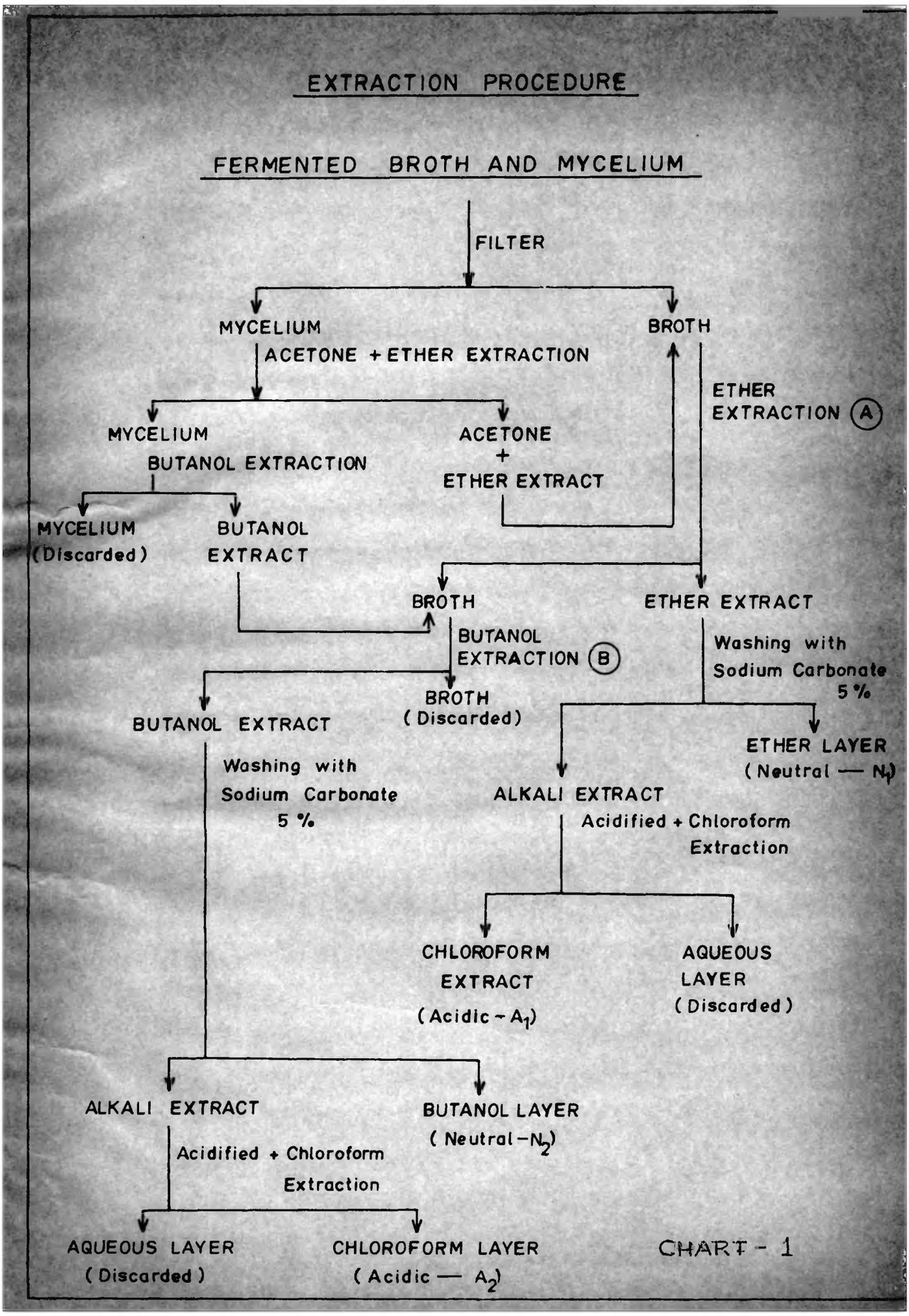


CHART - 1

The ether and butanol extracts were further washed with 5% sodium carbonate (50 ml x 3) to separate the acidic and neutral components. The neutral components were washed with brine, dried over sodium sulphate, concentrated and weighed. The sodium carbonate washings were then acidified and the acids extracted with chloroform (1/5th vol. x 3), washed, dried and weighed as described above.

(4) Separation

The neutral components were further separated by chromatography over alumina using the following sequence as a routine procedure : Pet-ether, Pet-ether : benzene; benzene, benzene : ether; ether, chloroform and finally with methanol with different proportions, excepting in the separation of products from bacterial fermentations, the use of benzene was omitted.

The acidic components were either converted into their methyl esters and further separated by chromatography over alumina or the acids were separated by fractional distillation.

(5) Identification

Each of the components was monitored by TLC or by VPC. The characterization of the compound was further achieved by physico-chemical methods such as I.R., n-m-r, etc.

[B] BACTERIAL TRANSFORMATION

(1) Maintenance and propagation of culture

The pseudomonad strain growing on limonene as the sole carbon source was propogated on a nutrient agar slant of the following composition (Mackie & McCartney, 1949).

Peptone (Constantino, Italy)	1 g
Sodium chloride (B.D.H.)	0.5 g
Yeast extract (Difco)	0.5 g
Beef extract (Difco)	0.5 g

made up to 100 ml

pH adjusted to 7.0 with 4N sodium hydroxide.

Each of the above items was dissolved separately and pooled together and the mixture made up to volume. For the preparation of the slants 6-8 ml of the medium were taken in 25 ml Pyrex or Borosil tubes and autoclaved at 15 psi (121°) for 20 minutes. These slants were inoculated from the broth or from the stock culture and incubated at 28° for 24 to 48 hr.

(2) Medium for growth

The organism was grown in a chemically defined medium according to Seubert (1960) having the following composition :

Dipotassium hydrogen phosphate (Danpha)	63.0 g
Potassium dihydrogen phosphate (Thomas Tyrer)	18.2 g
Ammonium nitrate (E.Merck)	10.0 g
Magnesium sulphate, anhydrous (B.D.H.)	1.0 g
Calcium chloride, $2H_2O$ (E.Merck)	1.0 g
Ferrous sulphate, $7H_2O$ (B.D.H.)	1.0 g
Manganese sulphate, anhydrous (Riedel-de Hain, A.G.)	6 mg
Sodium molybdate (B.D.H.)	6 mg

made up to 10 l.

pH 7.0

Aliquots of 100 ml medium were added to 500 ml Erlenmeyer flasks which were then autoclaved at 15 psi for 20 minutes. A 5 to 10% inoculum was added to sterilized medium. Aliquots of 0.3 to 0.5 ml of the terpenoid hydrocarbon were added and the flasks incubated at $28^{o} \pm 2^{o}$ on a rotary shaker (for 1-3 days). Fresh aliquots of 0.3 ml of terpenoid hydrocarbons were added at 24 hr intervals to compensate for the loss due to evaporation.

(3) Extraction

At the end of the incubation period the pooled culture from these flasks was acidified and the contents either centrifuged or first shaken up with ether (1/10 volume) to separate the bacterial mass from the broth. When centrifuged, the cells were extracted twice with acetone--ether solvent mixture (2 vols). The pooled extracts were slurried with hyflosupercel and filtered. The filtrate

was added to the broth and used for its extraction and followed up with three more portions of ether (1/10 vol. x 3). The bacterial cells with the hyflosupercel were further extracted with n-butanol (2 vols) and this butanol extract was used to re-extract the broth (1/10 volume x 2). After ether and butanol extraction, the cells were washed with water and the aqueous extract was added to the broth. The latter was rendered alkaline (pH 8.5 to 9.0) with ammonia (4N) and concentrated to a small volume (~ 100 ml) and was further extracted continuously with ether to recover the polar neutral fraction. The polar acidic fraction was recovered by acidifying the broth and further extraction with ether in a continuous extractor.

The ether and butanol extracts were subsequently separated into neutral and acidic fractions by washing with aqueous sodium carbonate (except in the case of the extract obtained in continuous extraction). The neutral components were further subjected to a four transfer Craig counter-current distribution between pet-ether and 90% methanol. (modified according to Prema and Bhattacharyya, 1962). Each fraction (non-polar and polar) was then subjected to chromatography over alumina and/or fractional distillation.

The acidic components were liberated by acidifying the alkaline aqueous extracts and extracting with chloroform. The residue after removal of chloroform was subjected to esterification with diazomethane in ether

and further separated into non-polar and polar fractions by a Craig counter current distribution as described above. The methyl esters (non-polar and polar) were ~~further~~ then subjected to chromatography over alumina and/or fractional distillation. The separation, isolation and identification of these compounds were monitored by physico-chemical methods such as TLC, VPC, n-m-r, and degradation studies as discussed in the relevant experimental sections.

[C] MANOMETRIC TECHNIQUES

Measurements of oxygen uptake of cell preparation and fractions were carried out by manometric techniques using Warburg flasks (25 ml capacity) with one side arm.

The total volume was 3 ml which consisted of 0.2 ml KOH in the centre well, 2.5 ml of cell suspension (3-10 mg dry wt) and 0.3 ml of 0.05 M phosphate buffer, pH 7.2 in the main compartment and ~ 20 µmoles of substrate in the side arm. The temperature was maintained at ~ 28^{o} unless otherwise stated.

CHAPTER - III

FERMENTATION OF LONGIFOLENE, CARYOPHYLLENE AND δ-CADINENE BY A. NIGER

FERMENTATION OF LONGIFOLENE, CARYOPHYLLENE AND δ-CADINENE BY ASPERGILLUS NIGER

DISCUSSION

Longifolene (30), $C_{15}H_{24}$, a tricyclic sesquiterpene occurs in the essential oil from the oleoresins of Pinus longifolia P-maritima. It is one of the main constituents of Indian turpentine oil, and was first isolated by Simonsen (1920). The structure of longifolene (30) was established from physico-chemical and degradation studies by Moffet and Rogers (1953) and Naffa and Ourisson (1953) and recently confirmed by Corey et al (1961) by its total synthesis.

The sample of longifolene employed in this experiment was prepared from Indian turpentine by a batch stripping distillation procedure and purified by distillation through a Tower's column under total reflux. However, from the recent work of Nayak and Sukh Dev (1963) it is probable that the sample also contained small amounts of longicycline, isolongifolene and longipinenes.

Caryophyllene (31), $C_{15}H_{24}$, This sesquiterpene forms the main constituent of oil of cloves (from Eugenia caryophyllata) and it occurs in African Copaiba oil (from Oxystigma mannii, Horm). An elaborate series of investigations led Deussen and Simonsen (1907) to conclude that

caryophyllene contains two or probably three hydrocarbons, α-caryophyllene, identical with humulene, β-caryophyllene and γ- or iso-caryophyllene. However, more recent work on caryophyllene seems to indicate that the oil of cloves contains only two sesquiterpene hydrocarbons, humulene and caryophyllene (Aibi et al, 1953; Lutz and Reid, 1953).

Robertson and Todd (1953) have carried out the X-ray crystallographic analysis of a halide of caryophyllene and established the stereochemistry of the halide. The total synthesis of caryophyllene has been achieved by William Brown (1959).

The hydrocarbon used in the present investigation was obtained from the B.D.H. It was purified in the usual way by batch stripping distillation process and had the physical constants reported in literature (Simonsen 1953) for pure caryophyllene.

δ-Cadinene (32), $C_{15}H_{24}$, This bicyclic hydrocarbon is distributed very widely in nature and was first isolated from the oil of cubebs by Sauberain and Capitaine (1840). The name cadinene was first proposed by Wallach (1887) who showed that it occurs also in a number of other essential oils (e.g. oil of ylang-ylang, citronella and sweet flag). The structure of cadinene has been established beyond doubt by the elegant experiments of Ruzicka and co-workers (1923) and Campell and Soffer (1942). Herout and Sykora (1958) have reviewed the chemistry of cadinenes

The sample of hydrocarbon was obtained from higher boiling fractions of Java citronella oil. The fraction distilling at $111.2^{o}/2$ mm was used in the present study and appeared to be a single component in the vapour phase chromatogram.

Fermentation. It was found in preliminary trials that longifolene can be easily metabolised by the strain of A.niger NCIM (612) at 0.1 to 0.2% levels. Fermentations were carried out both in shake flasks as well as in stainless steel fermentors of 6 l capacity equipped with arrangements for agitation and aeration. The products of fermentation were worked up according to the procedure of Prema and Bhattacharyya (1962) and separated into neutral and acidic components. The neutral component consisted mainly of unreacted longifolene and some mould fats and lipids and did not seem to contain any significant amount of neutral fraction. However, acidification of sodium carbonate layer and extraction of the acidified solution yielded a liquid anhydride on fractionation and purification.

This anhydride seemed to be identical with the compound isolated by Prema (1962) from the fermentation of camphene and β-santalene by the same strain of A.niger and gave the same imide and 2:4 dinitrophenyl hydrazine derivatives.

Structure of the anhydride

Prema (1962) established the structure of the anhydride by physico-chemical and degradative studies as well as by its synthesis. It is worthwhile to recapitulate some of the physico-chemical data at this stage.

Elementary analysis indicated the molecular formula, $C_{11}H_{16}O_3$. The neutral equivalent was 98 $\pm$ 2 which showed that the parent acid was a dibasic one. The I.R. spectrum (Fig.1) of the compound showed the presence of a five membered anhydride (1852, 1825, 1758 cm^{-1}), a tri or tetra substituted double bond (1668, 840 cm^{-1}) possibly conjugated with a carbonyl function and a straight chain containing at least four methylene groups (740 cm^{-1}). The UV spectrum (λ max. 249 mμ ; ϵ 6924) also supported the existence of an α,β-unsaturated carbonyl system in the molecule. The compound was optically inactive.

A Kuhn-Roth determination indicated the presence of two-methyl groups in the compound. By distillation of the diammonium salt of the free acid, a crystalline imide $C_{11}H_{17}O_2N$ (33) m.p. 60-61^{o} was obtained. The imide (33) showed characteristic I.R. absorption (Fig. 2) of a five membered ring imide (3200, 1768 and 1725 cm^{-1} (C = O) and the absence of the bands at 840 cm^{-1}). The liquid anhydride gave a mono-2:4,dinitrophenyl hydrazone m.p. 148-149^{o}, which showed UV absorption at 325 mμ

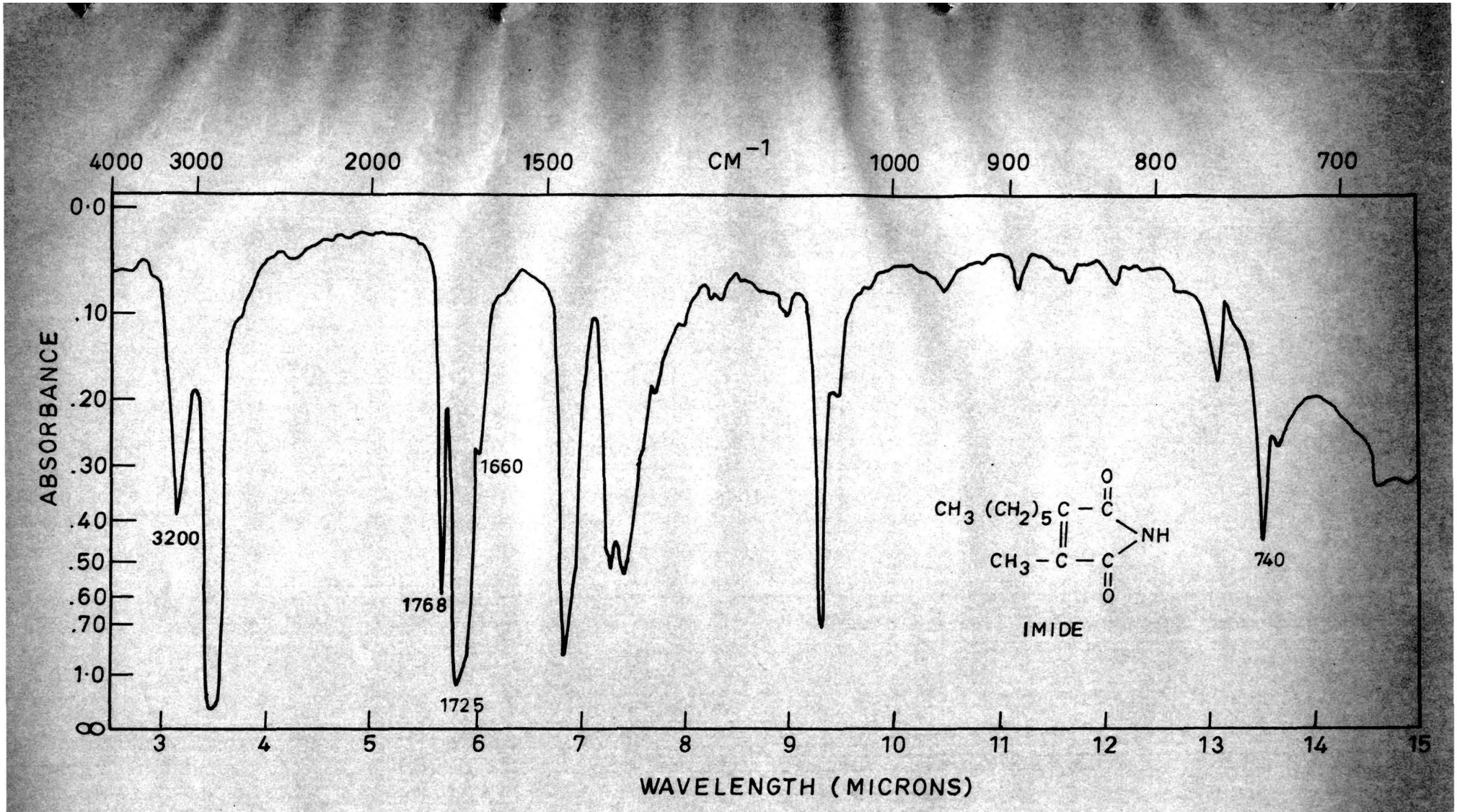

4000
3000
2000
1500
CM⁻¹
1000
900
800
700
0·0
.10
.20
.30
.40
.50
.60
.70
1·0
ABSORBANCE
3200
1768
1725
1660
740
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)
CH3 (CH2)5 C – C
CH3 – C – C
NH
O
IMIDE

FIG.- 2.

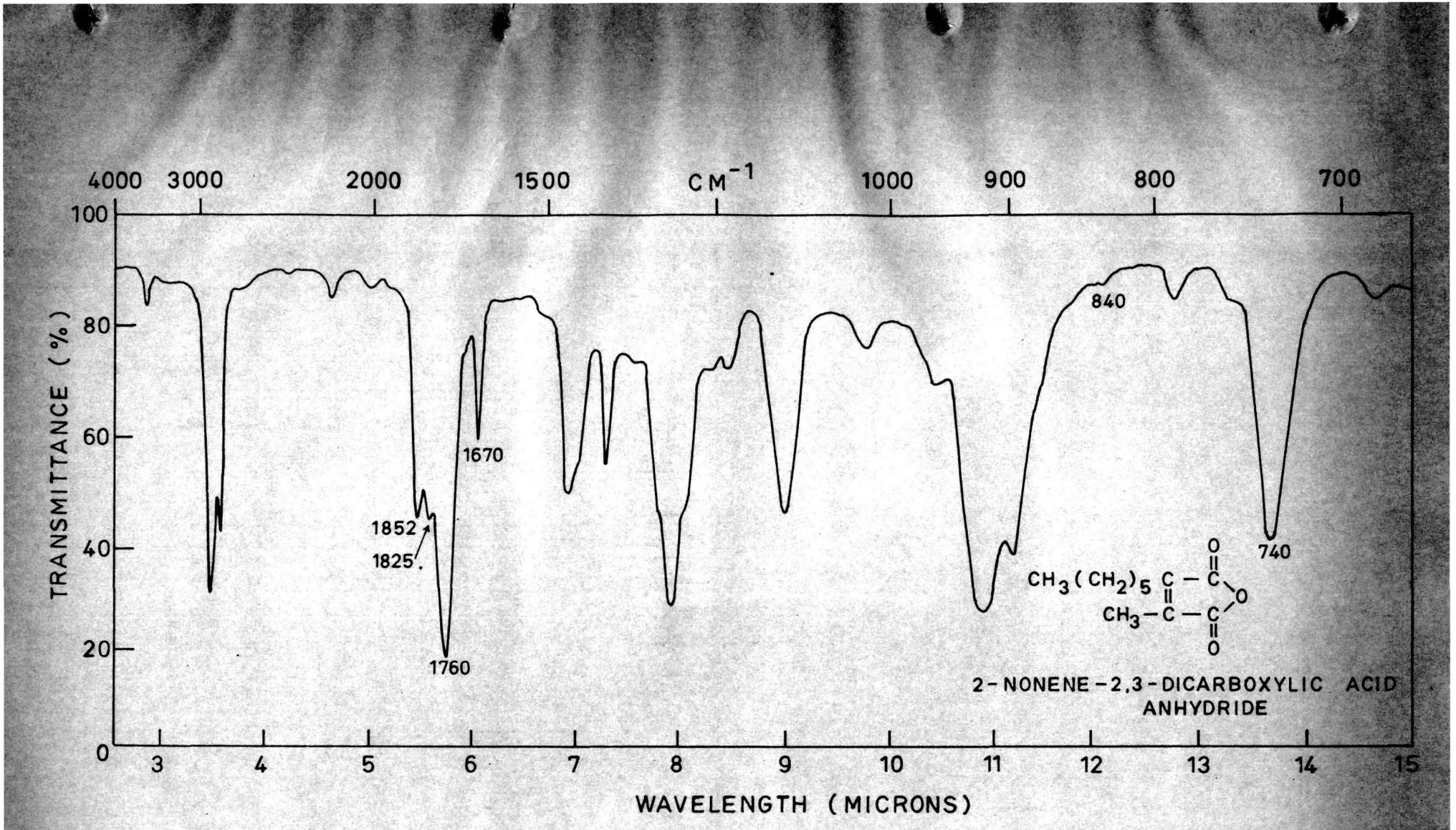
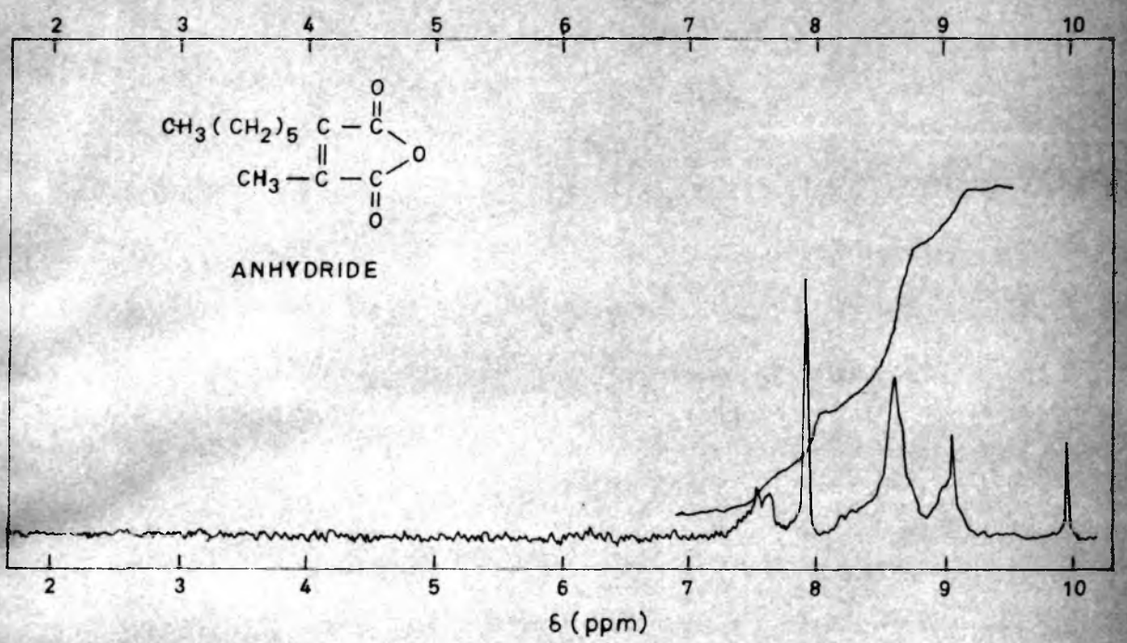

4000
3000
2000
1500
CM^{-1}
1000
900
800
700
100
80
60
40
20
0
TRANSMITTANCE (%)
1852
1825
1760
1670
840
740
$CH_3(CH_2)_5$ C — C
CH_3 — C — C
O
2-NONENE-2,3-DICARBOXYLIC ACID ANHYDRIDE
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

FIG.-1


2 3 4 5 6 7 8 9 10
ANHYDRIDE
2 3 4 5 6 7 8 9 10
δ (ppm)

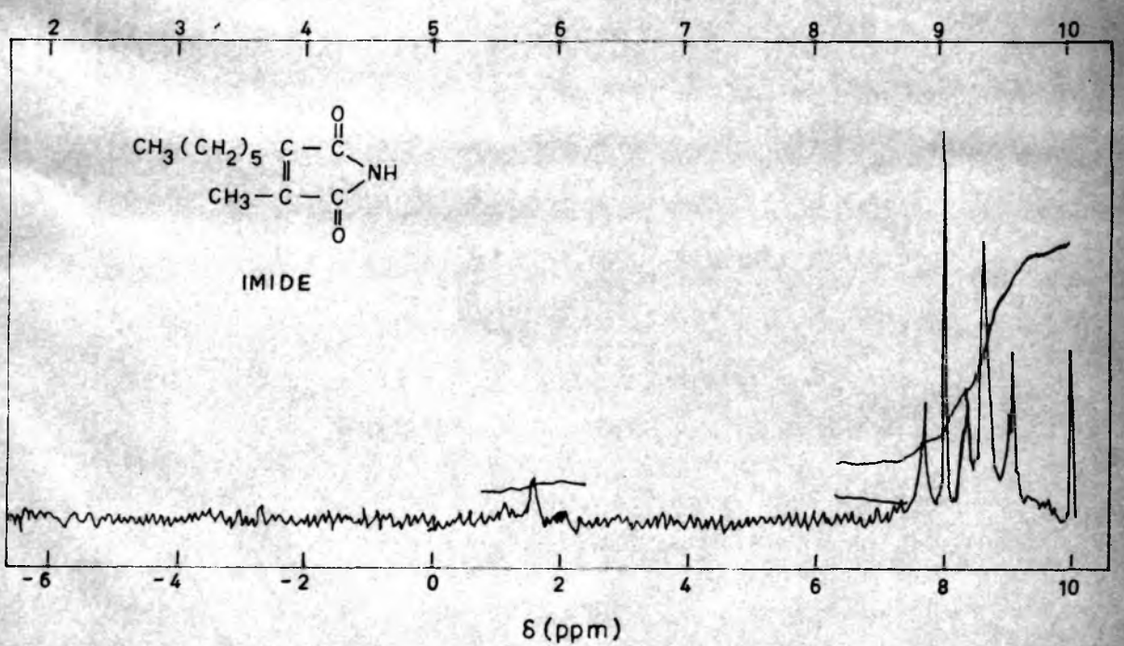

2 3 4 5 6 7 8 9 10
IMIDE
-6 -4 -2 0 2 4 6 8 10
δ (ppm)

FIG.-3.

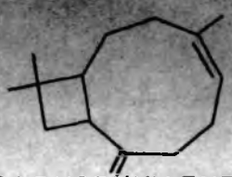
characteristic of a non-conjugated phenyl hydrazone indicating either (1) that the carbonyl reacting with phenyl hydrazine is not in conjugation with the double bond or (2) that the double bond might have shifted out of the conjugation during the formation of the derivative.

On the basis of the above findings the partial structure of the anhydride could be written as shown in Chart 2 , With the double bond in any one of the positions is indicated in dotted lines.

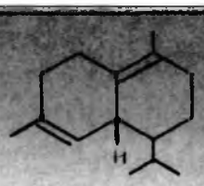
In the present work the presence of the double bond was clearly established by bromination of the anhydride to a dibromoderivative. It was also observed that on oxidation of the anhydride with chromium trioxide in acetic acid two volatile acids were isolated. These were identified as n-caproic and n-heptylic acids. When,however,the oxidation was carried out with sodium chromate in sulphuric acid, four volatile acids could be separated from the reaction mixture. These were established as acetic, propionic, n-caproic and n-heptylic acids (Chart 3) using paper chromatography.

These data confirm the structure of the anhydride (29) (Chart 2) which was derived by Prema (1962) by ozonolysis studies on the imide (Chart 2). The formation of the volatile acids can be explained by assuming an acid-catalysed equilibrium between structures (29) and (29a) (Chart 3).

LONGIFOLENE
(30)



CARYOPHYLLENE
(31)



6-CADINENE
(32)

$C_{11}H_{16}O_3$

2:4 dinitro phenyl hydrazine → $C_{17}H_{20}O_6N_4$ (m.p. 148°)

U.V. 325 mμ, ε 21,000

Kuhn roth - 2 $C-CH_3$ groups

Neu. Equi 98 ± 2

U.V. 249 mμ, ε 6,924

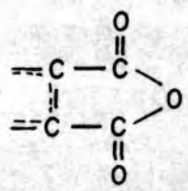
IR. 1) 1852, 1825 & 1758 CM^{-1}
2) 1668 & 840 CM^{-1}
3) 740 CM^{-1}

NH_4OH → $C_{11}H_{17}O_2N$ (m.p. 60-61°)

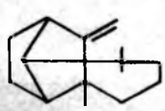
IR - 3200, 1668, 1725, 1660 CM^{-1}

U.V. 272 mμ, ε 5060

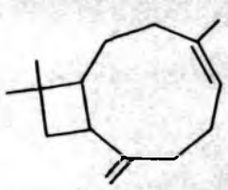
PARTIAL STRUCTURE



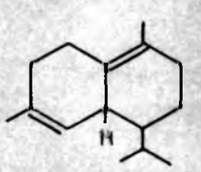
- Double bond
- C_7H_{16}
- 2 · C · CH_3
- $(CH_2)_4$ —



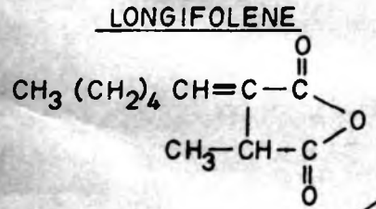
LONGIFOLENE



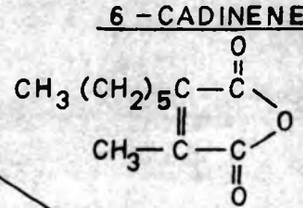
CARYOPHYLLENE



6-CADINENE

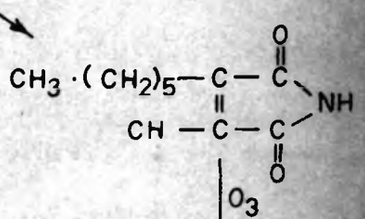


$C_{11}H_{16}O_3$

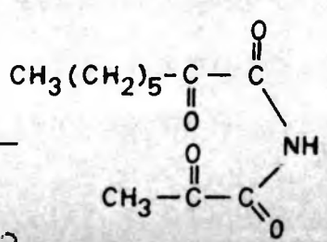


CrO_3 → $CH_3 \cdot (CH_2)_5 \cdot COOH$ + $CH_3(CH_2)_4 \cdot COOH$ + $CH_3 \cdot CH_2 \cdot COOH$ + $CH_3 \cdot COOH$

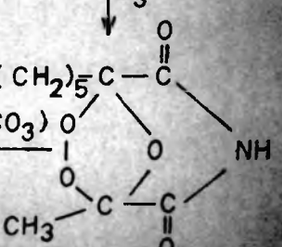
NH_4OH → $C_{11}H_{17}O_2N$



O_3



$Pd(CaCO_3)$ / H_2



HOH → $CH_3(CH_2)_5CO\ COOH$ + $CH_3 \cdot CO \cdot COOH$

CHART-2

Structure 29 can give rise to α-keto caprylic (34) and pyruvic acids (35) which would give n-heptylic (38) and acetic acids (39) on oxidative decarboxylation. Structure 29a can give rise to n-caproic acid (36) and α-methyl oxalacetic acid (37). The latter acid is presumably degraded to propionic acid (41) via α-keto butyric acid (40).

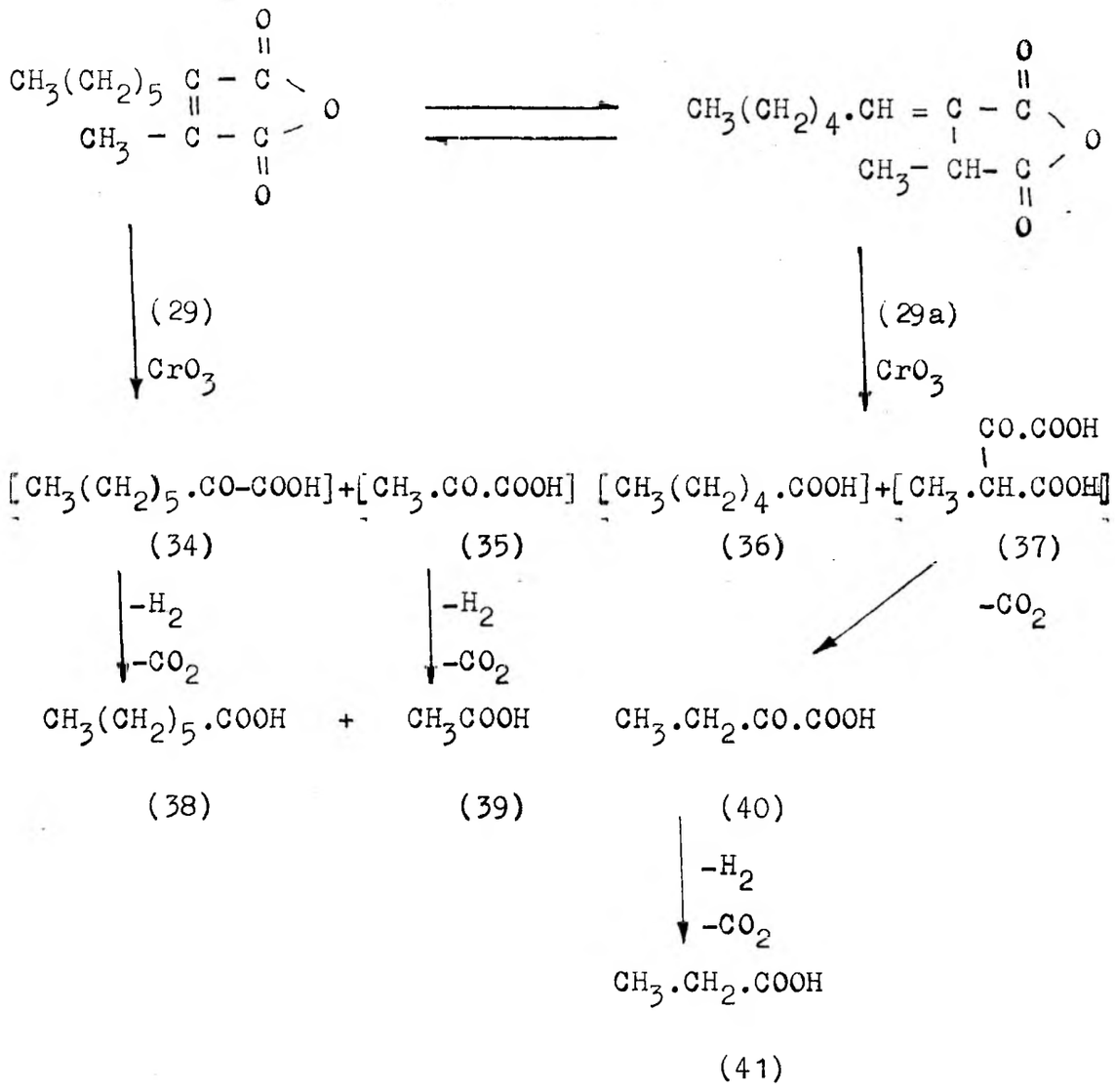


Chart-3

It may be mentioned in this connection that acid catalysed interconversions of citraconic and itaconic acids (Linstead et al, 1931) and their anhydrides (Barb, 1955) are known. The structure of the anhydride (29) and its imide (~~33~~) was further confirmed by their n-m-r spectra. It is seen from the spectra (Fig.3) that the vinyl proton is completely absent (absence of any bands between 2 τ and 6 τ). This rules out structure (29a).

Since the work on the structure of the anhydride from camphene and β-santalene on the one hand by Prema (1962) and from longifolene on the other hand was proceeding independently, it was considered necessary to develop an alternate synthesis for the anhydride. The first synthesis of the anhydride was reported by Prema (1962) consisted in a cyanohydrin synthesis of ethyl-α-n-hexylacetoacetate (42) as the first step followed by saponification of the cyanohydrin (43) and final dehydration of the hydroxy acid (44) (Chart 4). The yields were however, very poor.

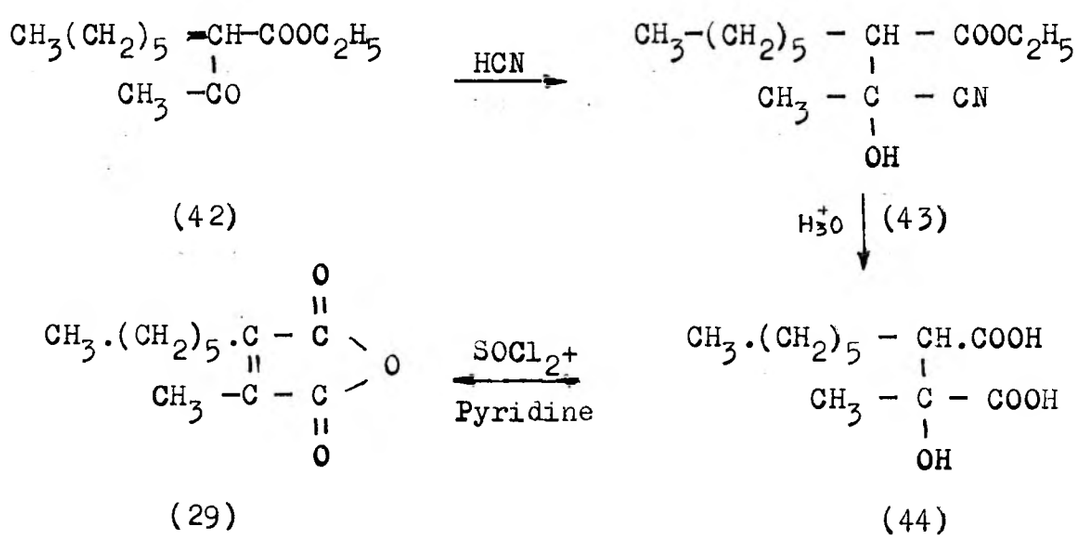


Chart-4

Better yields were obtained in the present work by a Stobbe-condensation of n-hexaldehyde (45) and dimethyl ester of α-methyl succinic acid (46) in the presence of potassium tert-butoxide. The resulting half ester (47) was hydrolysed to the dicarboxylic acid which yielded the desired anhydride on acidification (Chart 5).

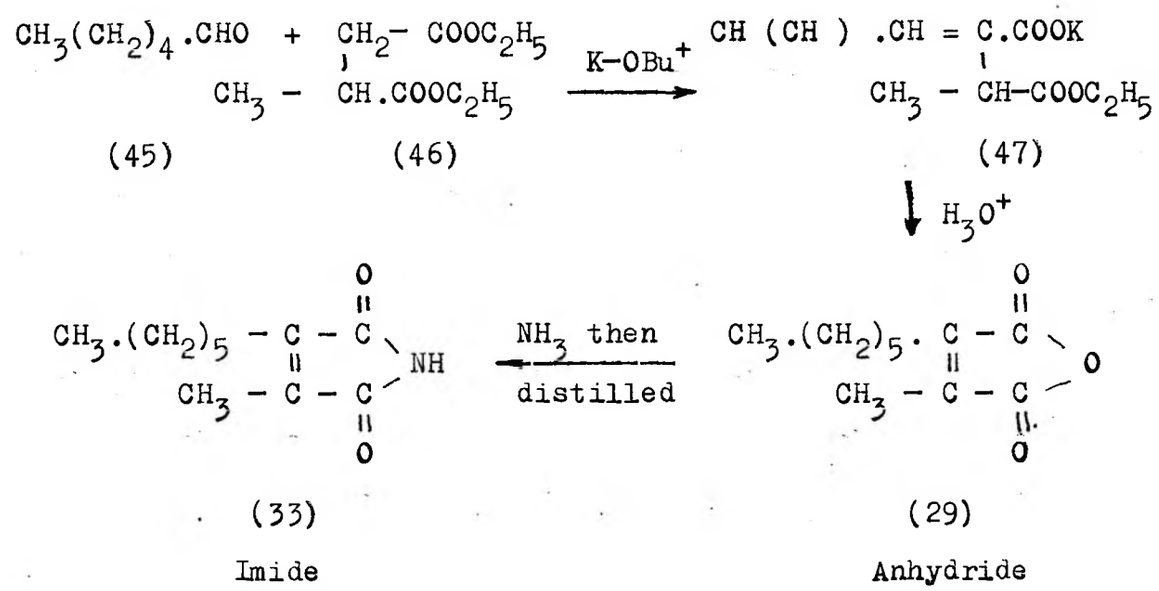


Chart-5

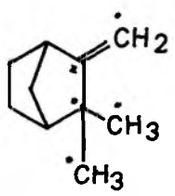
The anhydride (29) was converted to the imide (33) in the usual manner and both the synthetic anhydride and the corresponding imide was shown to have I.R.spectra identical with those of the isolated metabolite and its imide. There was no depression in the mixed m.p. of the two imides.

The origin of the anhydride 29

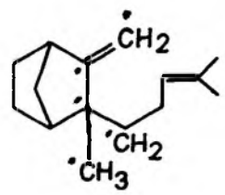
The structure of the anhydride bears no similarity whatsoever with that of any of the parent terpene hydrocarbons

used. Furthermore, camphene, which has ten carbon atoms also gives rise to the anhydride, which has eleven carbon atoms (Prema 1962).

Prema (1962) could not arrive at any definite conclusion regarding the origin of the anhydride. She, however, suggested the possibility that the carbon atoms of the citraconic acid moiety could have been derived from the *marked carbons of the terpenoid hydrocarbons as these are common to both the hydrocarbons.



CAMPHENE
(27)



β-SANTALENE
(28)

In her control experiments run without the hydrocarbon substrate, she could not detect the presence of the anhydride (29).

In the present studies it was found that the anhydride could be obtained in approximately 40% yields based on the initial longifolene. The rate of formation of the anhydride and the rate of disappearance of the neutral extractives were found to parallel each other (Fig.4), thereby indicating that longifolene takes part in the synthesis of the anhydride. It should also be noted that longifolene has also the (*) carbons which were implicated in the synthesis of the anhydride.

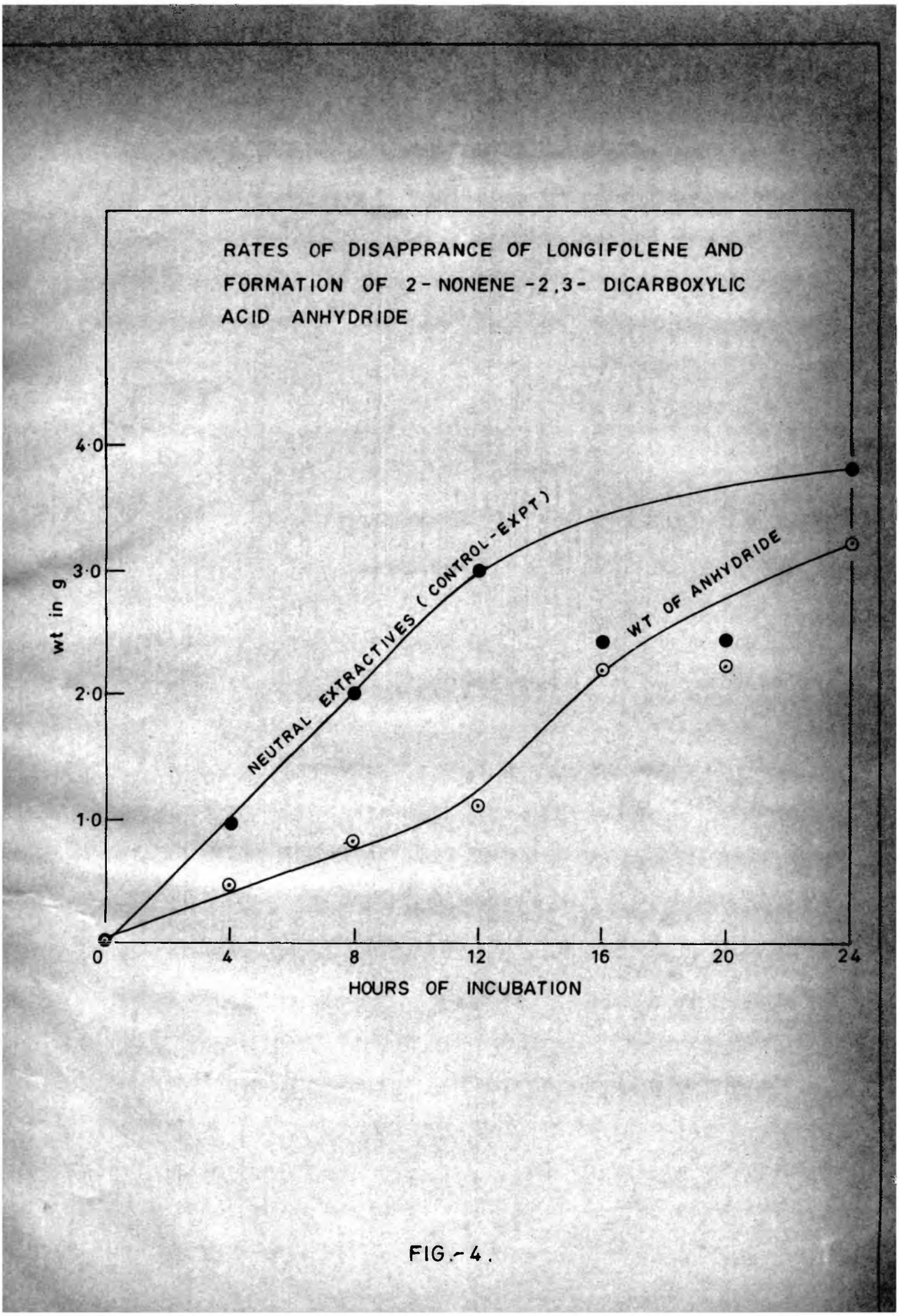

RATES OF DISAPPRANCE OF LONGIFOLENE AND FORMATION OF 2-NONENE-2,3- DICARBOXYLIC ACID ANHYDRIDE
wt in g
4·0
3·0
2·0
1·0
0
4
8
12
16
20
24
HOURS OF INCUBATION
NEUTRAL EXTRACTIVES (CONTROL-EXPT)
WT OF ANHYDRIDE

FIG.-4.

Fermentation of caryophyllene and δ-cadinene

At this stage it was discovered that two other structurally unrelated hydrocarbons, caryophyllene (31) and δ-cadinene (32) also gave rise to the same anhydride, when incubated with the same strain of A.niger. The preliminary working hypothesis of Prema (1962) that the citraconic carbons of the anhydride may be terpenoid in origin was rendered untenable by these new findings. It is not easy to visualise a similar arrangement of these five (*) carbons in caryophyllene (31) and δ-cadinene (32).

The only similarity in structure in four of the five hydrocarbons is an exocyclic methylenic double bond. It should be noted however that very little of the anhydride could be detected after incubation of β-pinene, another compound having the exocyclic methylenic double bond, with the strain of A.niger (vide Chapter IV).

These findings required a careful re-examination of the question of origin of the anhydride.

Paper chromatography

A suitable solvent system was developed to enable the identification of sub-micromole quantities of the anhydride by paper chromatography following the method of Reid and Lederer (1951). It was possible by this technique to detect the presence of small quantities of anhydride (29) in fermentation broth from controls run without the hydro-

carbon substrates. Following this lead it was possible even to isolate a small quantity of the anhydride (29) by pooling a few batches of controls and working up the extractives up in the usual manner.

It was, therefore, obvious that the anhydride is indeed a normal metabolite of the mould. It is produced in large quantities only where some terpenoid hydrocarbons are present in the fermentation mixture.

Now the question had to be settled whether or not any of the carbon skeletons of the terpenoid substrates are involved in the synthesis of the anhydride. For this radio-active tracers were employed.

TRACER STUDIES ON THE ORIGIN OF THE ANHYDRIDE

The fermentation of longifolene (30) was carried out in the presence of uniformly labelled glucose. The organism was allowed to grow for 24 to 30 hours on modified Czapeck-Dox medium in a special types of flasks. (Fig. 5).

Longifolene (30) and uniformly labelled glucose were added to each of the flasks and the fermentation was allowed to continue on the shaker under a continuous sweep of CO_2-free air at the rate of 3-4 bubbles/sec. The swept air was passed through two alkali bubbler traps connected in series to collect the respired $C^{14}O_2$.

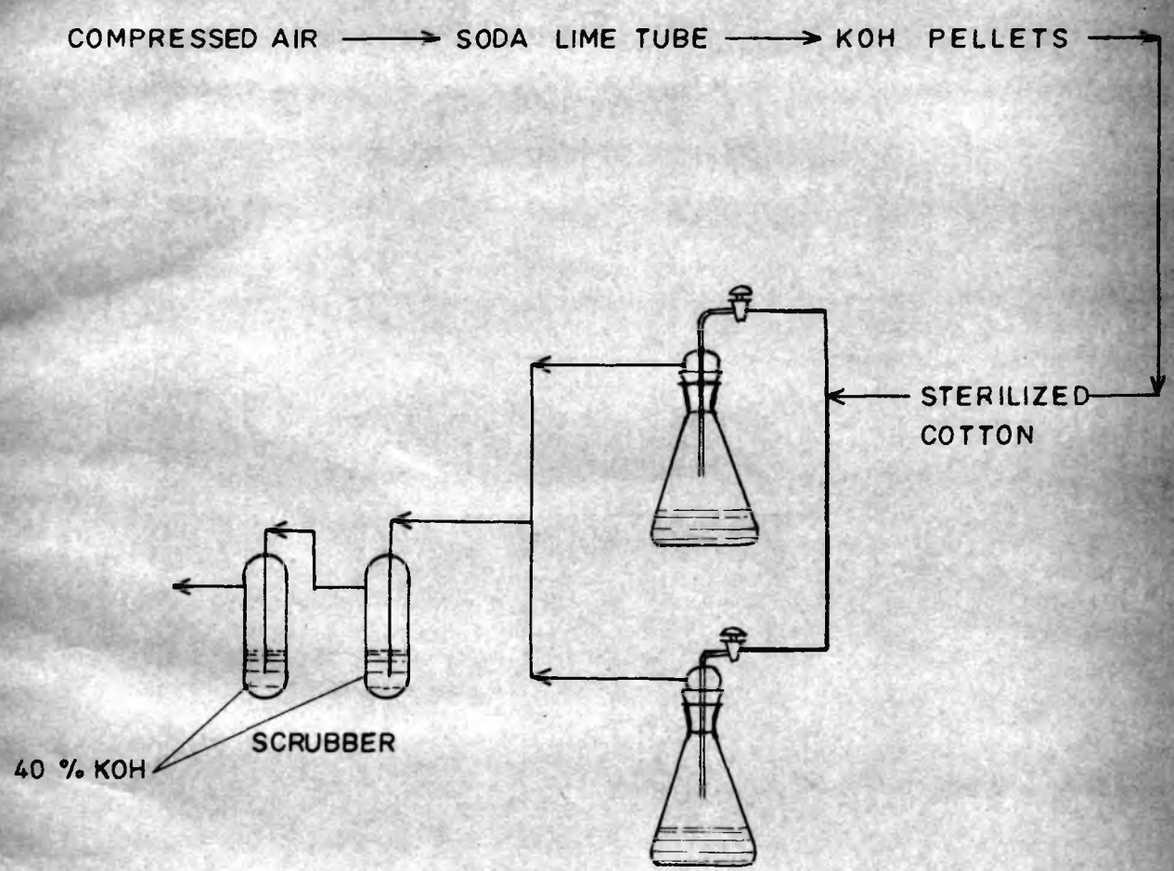

COMPRESSED AIR
SODA LIME TUBE
KOH PELLETS
STERILIZED
COTTON
SCRUBBER
40 % KOH

FIG. - 5.

After the fermentation, the contents of the flasks were pooled and worked up in the usual manner to obtain the crude anhydride (17 mg) which was converted to the imide by the dry distillation of the diammonium salt of the corresponding dicarboxylic acid (8-10 mg). The imide was purified by sublimation and was found to have a specific activity 120,000 cpm/mg . It was diluted six-fold with inactive imide.

The imide was then degraded by ozonolysis according to the following scheme - Chart 6 (based more or less on Prema's sequence of reactions, 1962) through the crystalline ozonide (48) and the diketoimide (49).

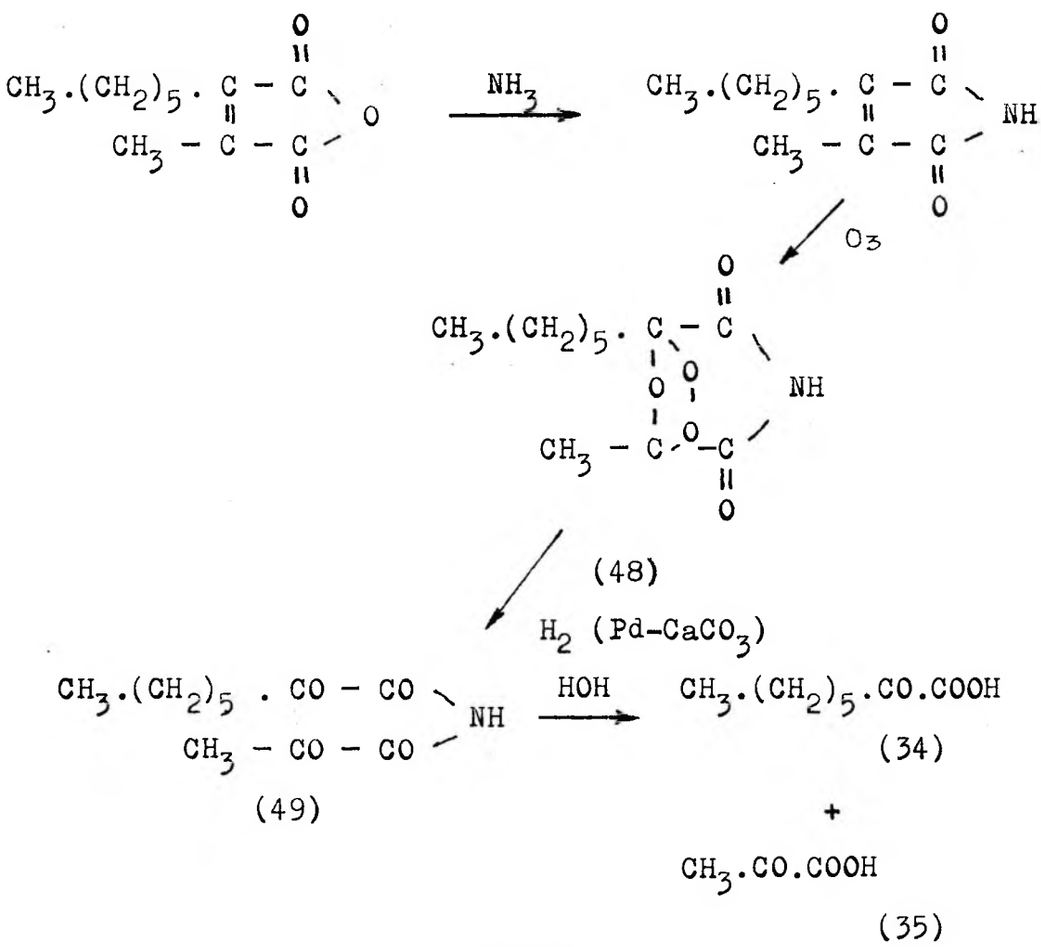


Chart-6

α-keto caprylic acid (34) and pyruvic acid (35) were separated by paper chromatography and the spots corresponding to α-keto caprylic acid and pyruvic acids were counted directly with a Tri-carb liquid Scintillation spectrometer using toluene as the reference fluid. The results (Table 1) indicate that both the fragments had approximately the same order of specific activity per micromole of carbon.

From these results it can be concluded that the anhydride is derived from the glucose in the medium and not from the terpenoid substrates. However, this experiment does not establish whether any of the terpenoid hydrocarbons participate in the synthesis of the anhydride through a metabolic pool of small carbon units. To varify this, it will be necessary to work with labelled longifolene and unlabelled glucose. Nevertheless, there appears to be no evidence of selective incorporation of the carbon skeleton from longifolene into any part of the anhydride. If the early working hypothesis of the origin of citraconic acid carbons from the terpenoid substrate were valid, then there could not have been any labelling of the pyruvic acid carbons.*

The origin of the anhydride appears, therefore, to lie in a possible condensation of octanoate or equivalent and pyruvate. It should be mentioned here that such condensation products of fatty acids, with some acids from the Kreb's cycle such as caperitic acid (50) (Asano et al, 1933) and roccellic acid (51) (Kennedy et al, 1937) have been isolated from lichens.

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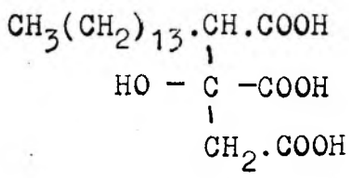
* In the present experiment the pyruvic acid carbons have a higher specific activity.

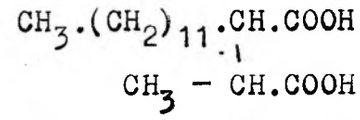
Specific activity of imide, α-keto-caprylic and pyruvic acids

Compound	Specific activity /m mole	Specific activity /μ m carbon
Imide $C_{11}H_{17}O_2N$	23,400	3140
α-Keto-caprylic acid $C_8H_{14}O_3$	15,250	3200
Pyruvic acid $C_3H_4O_3$	6,550	5300



Caperitic acid

(50)



roccellic acid

(51)

It appears probable that the presence of the terpenoid hydrocarbons impairs in some way in the normal fatty acid oxidation of the mould which releases anhydride as a detoxication product. This speculation however, remains to be supported by further isotopic as well as enzymatic studies.

It is noteworthy that all the hydrocarbons are oxidised by this mould, although they may not participate in the synthesis of the anhydride.

EXPERIMENTAL

[A] Determination of the optimum conditions for the fermentation.

(a) Concentration of substrate in the medium

It has been observed that the increase in the concentration of the substrate increases the transformation product. The maximum yields were about 40% when the concentration of the substrate was ~ 250-300 mg/100 ml of medium. With lower concentration of the substrate the yields were also low. (Table 2).

(b) Period of incubation

The period of incubation was also dependent on the conversion products and was proportional to the period of fermentation. With the fermentation period of 4 hr and 8 hr the formation of acidicproducts were 4.2 to 8% respectively and were proportionately increasing with the increase in the incubation period. With 20 to 24 hr incubation the yields of acidic transformation products were maximum and more or less steady. (Table 3).

(c) Temperature of conversion

The temperature was one of the most important and decisive factor in the fermentation with A.niger. With increase ($> 30^{o}$) in temperature though the growth of the

mould was profuse, the conversion was always negligible. With low temperature ($< 25^{\circ}$) the conversion is also low. The optimum temperature was found to be 28 ± 1 (Table 4).

Fermentation

The experimental conditions employed in the present investigation were essentially the same as described in chapter II - Methods and Materials. In the fermentations of longifolene (30), caryophyllene (31) and δ-cadinene (32) stainless steel fermentors were used with 4 l of medium. The mould under investigation was cultured in an energy rich synthetic modified Czapeck-Dox medium under aerobic conditions at 28° for 24 hr - 30 hr. The hydrocarbons longifolene (b.p. $254-256^{\circ}$, 712 mm, $[\alpha]_D$ + 37.5; 250 mg/100 ml medium), caryophyllene (b.p. $104-105^{\circ}/2$ mm; $[\alpha]_D^{28}$ - 9.7; 250 mg/100 ml medium) and δ-cadinene (b.p.111.2/2 mm; $[\alpha]_D$ + 46; 100 mg/100 ml) were added to respective cultures and incubated for a further period of 24 hr.

The method of extraction of the fermentation mixture was as described in chapter II - Materials and Methods. The products were separated into neutral (N_1) and acidic (A_1) components.

Table (2). Effect of concentration of longifolene on the formation of anhydride.

Concentration/ 4 l medium (g)	Incubation period (hr)	Unreacted longifolene (g)	Formation of anhydride (g)
4	24	3.3	0.5
8	"	5.2	2.5
12	"	7.0	4.0
16	"	13.0	2.8

Table (3). Effect of incubation period on the yields of 2-nonene, 2,3-dicarboxylic acid anhydride.

Time (hr)	Con. of longifolene /4 l medium (g)	Unreacted longifolene (g)	Formation of anhydride (g)
4	10	9.6	0.44
8	10	9.0	0.80
12	10	8.96	1.00
16	10	7.62	2.10
20	10	7.35	2.34
24	10	7.00	2.78

Table (4). Effect of temperature on the transformation of 2-nonene, 2,3-dicarboxylic acid anhydride.

Incubation period (hr)	Con. of longifolene /4 l medium (g)	Temperature	Unreacted longifolene (g)	Formation of anhydride (g)
24	10	25	8.0	1.5
24	10	28 ± 1	6.0	3.8
24	10	32	9.5	0.3

Products of fermentation of longifolene

The ethereal layer (N) containing the neutral fraction (N_1 - 6.2 gm) was dried over sodium sulphate, concentrated to a small volume (10 ml) and chromatographed over neutral alumina grade II (20 times the weight of the material to be separated). The solvent system used was pet-ether, benzene, ether, chloroform and methanol in different proportions as described in Chapter II - Materials and Methods. The pet ether fraction eluted unreacted longifolene (6 gm) and some amount of mould lipid, while benzene-ether (1:1) eluted out a solid alcohol (100-120 mg) which was characterized as ergosterol through mixed m.p. and comparative I.R. spectroscopy. Besides the unreacted longifolene, mould lipid and ergosterol, the neutral fraction did not give any hydroxylated or oxygenated products of the terpene.

The sodium carbonate layer (150-200 ml) containing the acidic products as the sodium salt was acidified with 6N HCl and extracted thrice with 60 ml portions of chloroform. The combined chloroform extracts after working up gave a highly viscous dark yellow oily liquid ($A_1 \sim 4$ gm) which was purified by repeated distillation under reduced pressure to yield the pure compound (b.p. 120-130° (bath)/3.5 mm).

The butanol extract (B) of the fermented medium did not give any significant amount of the conversion product.

The sesquiterpene caryophyllene (31) and δ-cadinene (32) also yielded the same compound i.e. 2-nonene-2,3-dicarboxylic acid anhydride (29) in 30 and 27% yields respectively from the sodium carbonate extract of the fermented broth and mycelium under identical conditions. In these cases also no other transformation products could be isolated from the neutral fraction.

Physico-chemical properties of compound (29)

B.p. 85-88° at 3 mm, $[\alpha]_D^{25} \pm 0$; neutral equivalent 98 ± 2 ; I.R. νmax, 1852, 1825, 1760, 1670, 1460, 1390, 1280, 1190, 1120, 1030, 965, 930, 904, 840, 790, 740, 690 cm^{-1} (Fig.1). UV, λ max Ether 249 mμ ,(ε , 6924). (Found : C, 67.19; H, 8.06. $C_{11}H_{16}O_3$; requires : C, 67.08; H, 8.12%). Kuhn-Roth determination : Found : $C\text{-}CH_3$, 27.20; $2(C\text{-}CH_3)$; requires : 27.0%) n-m-r spectrum (Fig.3).

Preparation of the imide (33)

The liquid anhydride (29) (750 mg) was dissolved in ice cold ammonia (sp. gr. 0.88; 4-5 ml) by shaking at 0°. After 30 minutes the excess ammonium hydroxide was removed in vacuo. The resultant ammonium salt was distilled carefully under reduced pressure (195-200° bath temp/1-1.5 mm) and the viscous colourless distillate (648 mg) allowed to solidify. The product was recrystallized from pet-ether

to give ~ 500 mg of the pure compound (33). The compound (33) can also be purified by sublimation under reduced pressure (55-57° at 3 mm). Colourless shining plates m.p. 61-62° U.V. λ max ^{Ether} 272 mμ (ϵ , 5060); $[\alpha]_D^{26} \pm 0$; I.R. νmax, 3200, 1768, 1725, 1660, 1463, 1370, 1350, 1300, 1070, 1052, 763, 740 cm^{-1}, (Fig.2). (Found : C, 67.39; H, 8.07; N, 6.86%; $C_{11}H_{17}O_2N$; requires : C, 67.66; H, 8.78; N, 7.17%) n-m-r spectrum (Fig.3).

The imide was converted to the anhydride according to the method of Prema (1962). The pure anhydride can be conveniently prepared in this way.

2:4-Dinitrophenylhydrazone of anhydride (29)

The anhydride (29) (120 mg) was added to a ethanolic solution (10 ml) of 2:4 dinitrophenyl hydrazine (100-125 mg) which contained 5-6 drops of concentrated sulphuric acid. The clear yellowish orange solution, thus obtained was left at 4-8° for 4-5 hours. A crude orange coloured crystalline product (180 mg) was obtained which had m.p. 144-145°. After recrystallization from dil. ethanol, brilliant yellow needles separated out which had m.p. 148-50°. The derivative of the anhydride was sublimed to give the purest analytical sample, m.p. 148-149°. U.V. λ max. 325 mμ (ϵ 21,000). I.R. νmax 3300, 1700, 1595, 1575, 1500, 1440, 1400, 1360, 1322, 1298, 1260, 1218, 1140, 1097, 1050, 913, 830, 753, 740, 730, 708, 680 cm^{-1} (not shown).

(Found : C, 53.7; H, 5.13; N, 14.69%; $C_{17}H_{20}N_4O_6$ requires : N, 14.89%).

Bromination of anhydride (29)

The anhydride (29) (500 mg) was dissolved in carbon tetrachloride (2 ml) and a volume of 2 ml of bromine in carbon tetrachloride (1:1) was added drop by drop with shaking. The contents were kept aside for 30 minutes. After removing the excess bromine, the residue was concentrated in vacuo . The compound obtained as a viscous gum. I.R. ν_{max} 1660 cm^{-1}, disappeared. (Found : Br, 46% for $C_{11}H_{16}O_3Br_2$; requires : Br, 47%).

Chromic acid oxidation of the anhydride (29)

The anhydride (29) (400 mg) was dissolved in 5 ml of glacial acetic acid and stirred with a mixture of sodium dichromate (3.5 g), sulphuric acid (6 ml) and water (8 ml) for 30 minutes at room temperature (20-25°). It was then refluxed for 30 minutes, cooled and treated with 20% aqueous solution of sodium sulphite (25 ml) to destroy excess chromic acid. The mixture was then steam distilled and the distillate was neutralized with ammonia and evaporated to dryness. Paper chromatography (Reid & Lederer, 1951) of the residue revealed the existence of four acidic components (RF 0.69, 0.63, 0.17 and 0.10) which were identified as n-heptylic (38), n-caproic (36), propionic (41) and acetic (39) acids respectively.

In a separate experiment, the anhydride (29) (0.5 g) was oxidized by stirring with chromium trioxide (1 g) in glacial acetic acid for 8 hours at 14-16°. The reaction was continued overnight at room temperature after which the excess chromic acid was destroyed by methanol (2 ml). The mixture was diluted with water (100 ml) and the acids were extracted in ether (50 ml x 3). After removing acetic acid from the ether extract by repeated washing with water, the other acids in the extract were re-extracted with 5% aqueous sodium carbonate (25 ml x 2). After acidification of the carbonate solution the acids were extracted by chloroform. The chloroform extract was washed, dried and evaporated to yield a mixture of liquid acids (358 mg), which was subjected to fractional distillation at 0.2 mm. The first fraction (200 mg) which collected at 100-110° (bath temp.) consisted mainly of n-caproic acid (36) as revealed by paper chromatography (Reid & Lederer, 1951). Neutralization equi. 114 ± 4; I.R. ν_{max}. 2600, 1680, 1440, 1390, 1360, 1260, 1230, 1195, 1160, 1095, 1040, 960, 820, 730 cm^{-1}. (Found : C, 61.4; H, 10.5% for $C_6H_{12}O_2$ requires : C, 62.0; H, 10.4%) ~~(not shown)~~; anilide m.p. 94°; literature (for n-caproic acid anilide m.p. 95°) (Heilbron, 1946a)

The second fraction (100 mg) distilled at 120° (bath temp.) was mainly n-heptylic acid (38) as established by its conversion to its anilide by the usual method. Re-crystallized from ethanol; m.p. 70° (reported for

heptylic acid anilide) (Heilbron, 1946b) (70-71°) mixed m.p. 69-71° with authentic sample. (Found : C, 76.9; H, 9.3; N, 7.2%; $C_{13}H_{19}NO$ requires : C, 76.1, H, 9.3; N, 6.8%). The I.R. spectra of both the C_7 acid and its anilide were identical with those of the authentic sample.

Total synthesis of the anhydride (29)

n-Hexaldehyde (45) (1.04 g) and diethyl-α-methyl succinate (46) (1.88 g) were added to a solution of metallic potassium (0.34 g) in tert-butanol (8 ml) under an atmosphere of nitrogen in an oven dried apparatus. The mixture was refluxed under vigorous stirring for 14 hours in a nitrogen atmosphere. It was then decomposed with the careful addition of crushed ice and the crude half ester (47) was hydrolysed for 1 hour on a steam bath. After acidification the anhydride was extracted in the usual manner with ether (10 ml x 3) and purified by distillation (yield 68 mg). The material had identical infrared spectrum with that of the natural product. (Found : C, 67.1; H, 8.62%; $C_{11}H_{16}O_3$ requires : C, 67.08; H, 8.12%). The imide (33) was prepared by the ammonia method; m.p. 60-61°. The physico-chemical properties of this synthetic imide and its I.R. spectrum with those of the corresponding derivatives of the natural product were identical. There was no depression in the mixed m.p.

Control experiments without substrate

In these experiments conditions employed were essentially the same as described earlier except that the substrate was not added. The contents of the stainless steel fermentor were removed, extracted and worked up as before. From the acidic fraction 30-35 mg (pooled from two fermentors) of the compound was isolated which was shown to have 2-nonene-2:3-dicarboxylic acid anhydride on paper chromatograms using butanol -NH_3 water as the developing solvent system, (R_F 0.55) and bromophenol blue as the spraying reagent.

Tracer studies

To each of two 500 ml special flasks (Fig.5) containing 100 ml each of modified Czapeck-Dox medium previously sterilized at 15 psi pressure for 20 minutes were inoculated with A.niger NCIM 612 and incubated for 24 hr. Aliquots of 0.7 ml of glucose -U-C^{14} (activity 0.033 mc; sp.activity 84 mc/m mole) containing 0.2 ml longifolene was transferred into each flask and the incubation was continued for another 24 hours under a continuous sweep with CO_2-free air, at the rate of 3-4 bubbles/sec. The swept air was passed through two alkali traps connected in series to collect the respired $C^{14}O_2$.

After the fermentation the contents of the flasks were pooled and worked up in the usual manner to obtain the crude anhydride (29) (17 mg) which was converted to the imide (33) by distilling the diammonium salt of the corresponding dicarboxylic acid (8-10 mg). The imide was purified by sublimation (m.p. 61-62°). An aliquot of 3 mg of the imide was diluted with 15 mg of inactive imide and dissolved in 10 ml ethyl acetate. An aliquot 0.5 ml was removed and its activity was determined separately. The rest of the solution was used for ozonolysis.

Ozonolysis of the imide

The imide (17.1 mg in 9.5 ml ethyl acetate) was ozonolysed for two hours (starch iodide test). After the ozonolysis was complete, the ozonide (48) (20 mg), without removing the solvent was hydrogenated, for 2 hours using 5% active palladium over calcium carbonate as a catalyst. The reaction mixture was filtered through Hyflo-supercel. The residue was washed with ethyl acetate and the filtrate and washings were combined and evaporated to isolate the diketoimide (49) (17 mg).

To the diketoimide (17 mg) saturated barium hydroxide (5 ml) was added and the contents were shaken at room temperature for 24 hr. Barium hydroxide was decomposed by the slow addition of a slight excess of dilute sulphuric acid (0.5 ml of 4N). The mixture was filtered and the

filtrate extracted with chloroform (5 ml x 4). The chloroform extract after working up gave a liquid product which was redissolved in 5 ml of fresh chloroform. The aqueous extract was kept separately.

2:4-Dinitrophenyl hydrozone of the chloroform extract

To hot methanol (1 ml) containing a drop of sulphuric acid an aliquot of the chloroform extract (2 ml) was added and the contents left in the cold for 3-4 hr. The precipitated 2,4-dinitrophenyl hydrazone was filtered and washed with cold methanol (0.3 ml). The crude DNP-derivatives of the two acids were separated by fractional crystallization. They were forced to have m.p. 126-128° and 210-215° respectively.

Paper chromatography of the DNP acids

The DNP derivatives of α-keto caprylic and pyruvic acids were chromatographed for 20 hr on a Whatman filter paper No.1 using butanol-formic acid saturated with water (95 ml butanol, 5 ml, formic acid) as the developing solvent. Authentic samples of DNP α-keto caprylic acid and pyruvic acid were also spotted along with the unknowns. The two spots (R_F 0.88, 0.55) which showed mobilities identical with dinitrophenyl hydrazones of authentic samples of α-keto-caprylic and pyruvic acids, which were cut and counted separately on a Tricarb scintillation counter.

However due to quenching effect of the scintillation the activity of the DNP derivative could not be accurately determined.

Paper chromatography of the free keto acids (34 and 35)

The chloroform extract containing the free acids was chromatographed on Whatman filter paper No.1 with authentic samples of α-keto caprylic and pyruvic acids using butanol-formic acid-water (95:5 sat. with water) as the developing solvent system. The chromatogram was air dried and the excess formic acid was removed by exposing the chromatogram to a steam bath. The spots corresponding to α-keto caprylic and pyruvic acids were made visible under UV light and were cut out and directly estimated using a scintillation fluid on a Tricarb scintillation counter. The α-keto caprylic acid had the activity 63.0 cpm. while pyruvic acid had the activity 27 cpm. The specific activities of these are reported in Table 1.

CHAPTER - IV

TRANSFORMATION OF β-PINENE

TRANSFORMATION OF β-PINENE

DISCUSSION

In the preceding chapter the conversion of longifolene (30), caryophyllene (31) and δ-cadinene (32) by the experimental strain of A.niger NCIM (612) was described. It was considered necessary to determine whether (like camphene (27) and β-santalene (28)) β-pinene (52) also gives rise to 2-nonene-2,3-dicarboxylic acid anhydride, as it also has a exocyclic methylenic double bond in the molecule similar to that in longifolene.

β-pinene, $C_{10}H_{16}$ (52), also known as nopinine, is found in most essential oils which contain α-pinene but in much smaller proportions, only the (+) form being detected in Ferula galbanifluo and in Cyanamoranthum mittallii . The structure of the hydrocarbon was deduced from the hydroxy acid which has been named nopinic acid. (Rodd, Barton, 1953).

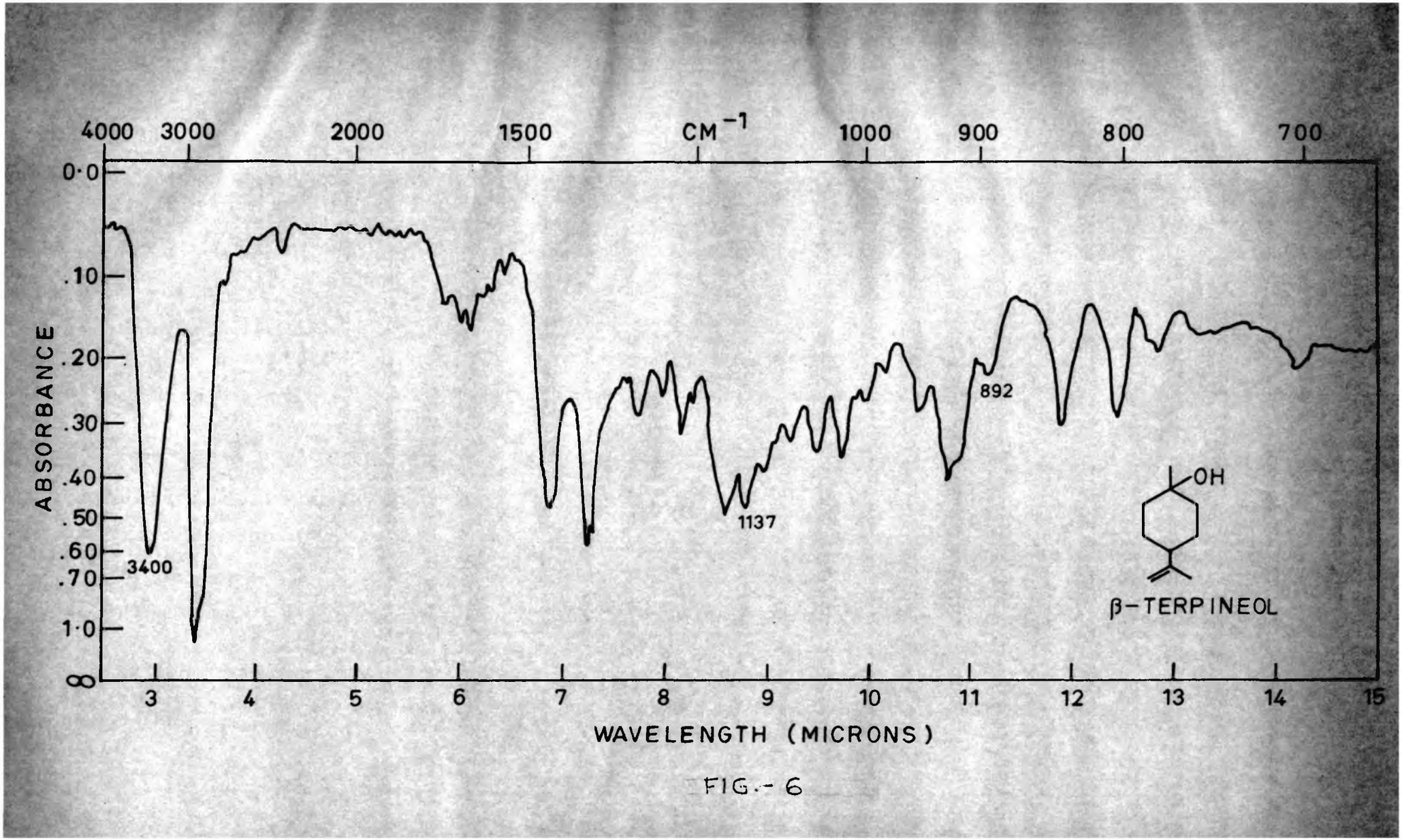
The synthesis of β-pinene was achieved by Bonnet et al (1938).

When β-pinene (52) was incubated with A.niger (612) only trace amounts of the anhydride was formed. However, the neutral fraction in this case contained several transformation products from which the major component was isolated as the monohydroxy compound.

Fermentation conditions and the extraction procedure were essentially the same as those used with α-pinene by Prema (1962). The ether extract was washed with 5% sodium carbonate and separated into acidic (A) and neutral (N) components. The latter which contained the unreacted and the oxygenated products of β-pinene were subjected to four transfer distributions between light petroleum and 90% aqueous methanol. The light petroleum extract (N - I) containing the unreacted β-pinene and non-polar oxygenated components and the 90% methanol extract (N - II) containing the polar-oxygenated components were further subjected to chromatography over alumina.

The fraction (N-I) when eluted with pet-ether gave the unreacted hydrocarbon, fat and other lipids in the eluate. Further elution with a mixture of pet-ether and ether gave a solid compound (m.p. 158-159°) which was identified as ergosterol by comparative I.R. spectra and a mixed m.p. determination.

The methanol fraction (N-II) when chromatographed over alumina with benzene-ether gave a sweet smelling liquid. The compound showed a single spot on TLC. The I.R.spectrum of this compound (Fig. 6) showed a hydroxy group ( )max 3400, 1137 cm^{-1}) and an isobutylenic double bond (892 cm^{-1}). The elementary analysis of this compound agreed well with the molecular formula $C_{10}H_{18}O$. Attempts to acetylate with acetic anhydride and pyridine at room temperature were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3000
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1500
CM -1
1000
900
800
700
0·0
.10
.20
.30
.40
.50
.60
.70
1·0
ABSORBANCE
3400
1137
892
OH
β-TERPINEOL
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

FIG.- 6

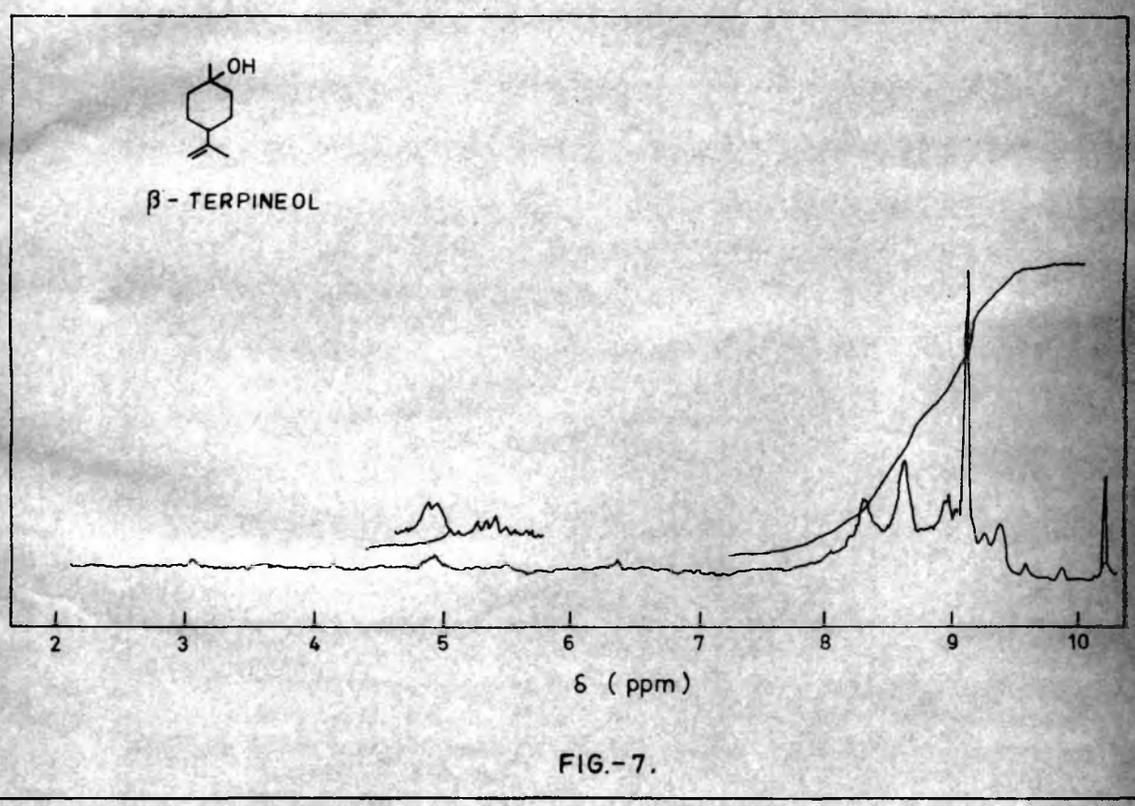


FIG.-7.

unsuccessful indicating that the hydroxyl group is not primary or secondary.

The n-m-r spectrum of the compound (53) (Fig. 7) indicated the presence of an isobutylenic side-chain (methyl-proton signal at 8.4 τ and methylene protons at 5.38 τ). The other methyl signal was found as a sharp peak at 8.87 τ indicating that the carbon atom probably carried an oxygen function. These data indicated that the compound may be β-terpineol (53).

When the monohydroxy compound (53) was treated with phenyl isocyanate, a white crystalline derivative (m.p. 80-82°) was obtained. This phenyl urethane of this compound was found to be identical with that of β-terpineol which melts at 83-85° (Perkin 1904). This confirms that the compound (53) is β-terpineol. It was also observed that traces of β-terpineol were obtained in control experiments without the mould. However, the yields were negligible, being of the order of 2% of the yields obtained in the experiments with the mould.

It is interesting to note that unlike α-pinene which gave rise to oxidation products after fermentation with the same strain of A.niger (Prema and Bhattacharyya 1962a) the isomeric β-terpene only gave rise to a hydration product - β-terpineol.

EXPERIMENTAL

β-pinene (52) used in the present investigation was a B.D.H. product. It was purified by distilling by a batch stripping process and further purified by passing over alumina grade I. It distilled at 163-164°/711 mm, n_D^{25}, 1.4800; $[\alpha]_D$ - 22°. It showed a single peak on VPC with retention time 5 minutes at 106°, 10 sec/10ml, 163 m.amp., polyester of succinic acid.

Twenty flasks each containing 100 ml modified Czapeck-Dox medium were sterilized at 15 psi pressure for 20 minutes. The content of each flask was inoculated with A.niger (612), spores from PDA slants grown for a week at 28°. The mycelium was allowed to grow for 24 hr. The substrate β-pinene was added at the level of 0.3 ml/100 ml medium and the fermentation was allowed to proceed for 8 hr. At the end of this period another lot of β-pinene (0.2 ml per flask) was added and the fermentation continued for another 8 hours. The contents were then removed, pooled and extracted in the usual manner described earlier under 'Materials and Methods' (chapter II).

The acidic (A) and neutral (N) fractions were separated by 5% sodium carbonate extraction and the neutral fraction (N - 7.3 g) was subjected to 4-transfer distributions between pet-ether and 90% aqueous methanol. The pet-ether fraction (N-I 6.7 g) after working up was subjected to chromatography over alumina (120 g).

Chromatography of neutral fraction (non-polar - N-I, 6.7 g)

The first fraction eluted with pet-ether was found to consist of unreacted β-pinene (6.6 g) and a small amount (20 mg) of lipid material. The second fraction eluting out in pet-ether containing 10% ether was a solid (m.p. 158° - 25 mg) which was identified as ergosterol by comparative I.R. and mixed m.p. with authentic sample.

Apart from unreacted β-pinene no oxygenated conversion products of β-pinene could be detected.

Chromatography of neutral fraction (polar, N-II, 490 mg)

The neutral polar fraction (490 mg) was chromatographed over alumina (10 g).

The first fraction eluted with pet-ether consisted of only some lipid material.

Successive elution with benzene containing increasing amounts of ether from 10 to 75% liberated a liquid compound (200 mg) which showed practically a single spot in TLC (20% ethyl acetate in hexane, R_F 0.45). It was purified by distillation in vacuo, b.p. 105-110° (bath) at 4 mm or 75-78°/4 mm. $[\alpha]_D^{27}$ - 52.5°, I.R. ν_{max} 3400, 1645, 1450, 1375, 1290, 1160, 1137, 1080, 1052, 1025, 1000, 952, 928, 918, 892, 840, 802, 780, 704 cm^{-1} (Fig. 6) (Found : C, 78.0, H, 10.95%; calculated for $C_{10}H_{18}O$; C, 77.86, H, 11.76%).

Preparation of phenyl urethane of compound (33)

The monohydroxy compound (33) (20 mg) was treated with phenyl isocyanate (0.5 ml) in the cold. The mixture was allowed to remain at room temperature overnight after which it was kept in a steam bath for a while. Crystallization was induced by scratching with a glass rod. The mixture was then extracted with hot hexane (2 ml). The extract on concentration and cooling gave a crystalline solid (m.p. 80-82°) (Found : C, 74.5, H, 9.2%; calculated for $C_{17}H_{25}O_2N$; C, 74.14, H, 9.15%). β-terpineol urethane melts at 83-85°.

Apart from the above compound small amounts of other hydroxy compounds were also obtained from the neutral polar fraction. The quantities were, however, too small for their isolation and characterization.

Acidic fraction (A)

The acidic fraction (200 mg) on treatment with diazomethane gave 203 mg methyl ester. This ester after saponication with 2 ml of 4N potassium hydroxide in ethanol and keeping overnight followed by dilution, acidification and extraction with ether (10 ml x 3) yielded a compound (30 mg) which was identified as 2-nonene-2,3-dicarboxylic acid anhydride (29) by comparative I.R.spectra and paper chromatography.

Auto-oxidation of β-pinene

The conditions for incubation were the same except that the mould was not added to the flasks. Neutral fraction yielded only 11 mg from which 4 mg of β-terpineol fraction was obtained by chromatography and identified from VPC.

CHAPTER - V

BACTERIAL TRANSFORMATIONS OF LIMONENE

BACTERIAL TRANSFORMATION OF LIMONENE

DISCUSSION

Working with fungi in the transformations of terpenes is disadvantageous in some respects. In the first place it is not possible to grow the fungi with the terpenoid hydrocarbons as the sole source of carbon. The fermentation mixture with these micro-organisms which are first grown in an energy rich medium and then treated with the hydrocarbon contains other metabolites of non-terpenoid origin. The formation of 2-nonene-2,3-dicarboxylic acid anhydride as discussed in the earlier chapter is a case in point. In the second place, manometric studies with fungal pellets indicate that in almost all cases the oxygen uptake on these hydrocarbons as substrates is not very significantly higher than the endogenous respiration. In the third place attempts at extraction of some of the terpene-transforming enzymes from the moulds resulted in the preparations containing little or no activity.

It was, therefore decided to initiate a search for a suitable bacterium which can possibly utilise terpenoid hydrocarbons as its sole source of carbon. A soil pseudomonad capable of growing on limonene as the sole organic-substrate was isolated by enrichment culture techniques. The characterization as well as studies on the microscopic

and the biochemical performances of the organism are presented in this chapter.

Limonene, $C_{10}H_{16}$ (55); this monoterpene occurs very widely distributed in nature and has played a fundamental part in the development of terpene chemistry. Limonene forms the main constituent of terpene fraction of number of oils such as the oils of lemon, orange, etc. (SIMONSEN, 1953)

Besides limonene, this bacterium grows also easily on β-pinene and p-cymene but with difficulty on α-pinene. It utilizes the Krebs cycle acids such as succinate, malate and acetate. Glucose, maltose, arabinose and sucrose are also utilized by the organism. The ready growth of this bacterium on the members of the tricarboxylic acid cycle would indicate that all the Krebs cycle enzymes are present in this bacterium. A detailed enzymic analysis was not undertaken, since such analyses would be beyond the scope of the current work.

The organism grows quite rapidly on limonene, the rate being comparable to that of glucose. The growth of this bacterium on various substrates is indicated in the experimental section. Preliminary manometric studies were indicative of a complete oxidation of a major part of limonene to carbon dioxide and water according to the following equation.

$$C_{10}H_{16} + 14O_2 \longrightarrow 10CO_2 + 8H_2O$$

However the total oxygen uptake never reached upto the theoretical maximum and this indicated that some intermediates might accumulate in the medium.

It was necessary to find out the optimum substrate concentration to get the maximum amount of growth. After trial experiments at different graded levels of substrates, the concentration of 0.3 to 0.4 ml per 100 ml medium was decided upon for the preparative scale fermentation with 40 shake flasks at a time.

Fermentation of limonene (55)

The extraction procedure after the fermentation was more or less the same as that used for fungal fermentations and is summarised in chart 7 and detailed in the chapter on Materials and Methods. Briefly, the broth was acidified and extracted with ether. Hyflosupercel was added to the etherial layer containing the bacterial cells. The hyflo-supercel cakes containing the bacterial cells were also extracted first with a little acetone ether and then with ether. The residual bacterial cells and the filtrate were further extracted with n-butanol. Finally after the butanol extract, the hyflosupercel containing bacterial cells was extracted with water and the filtrate after neutralizing with ammonia was concentrated and further extracted with ether in a continuous extractor to recover the most polar, neutral and acidic, transformation products. The extracts

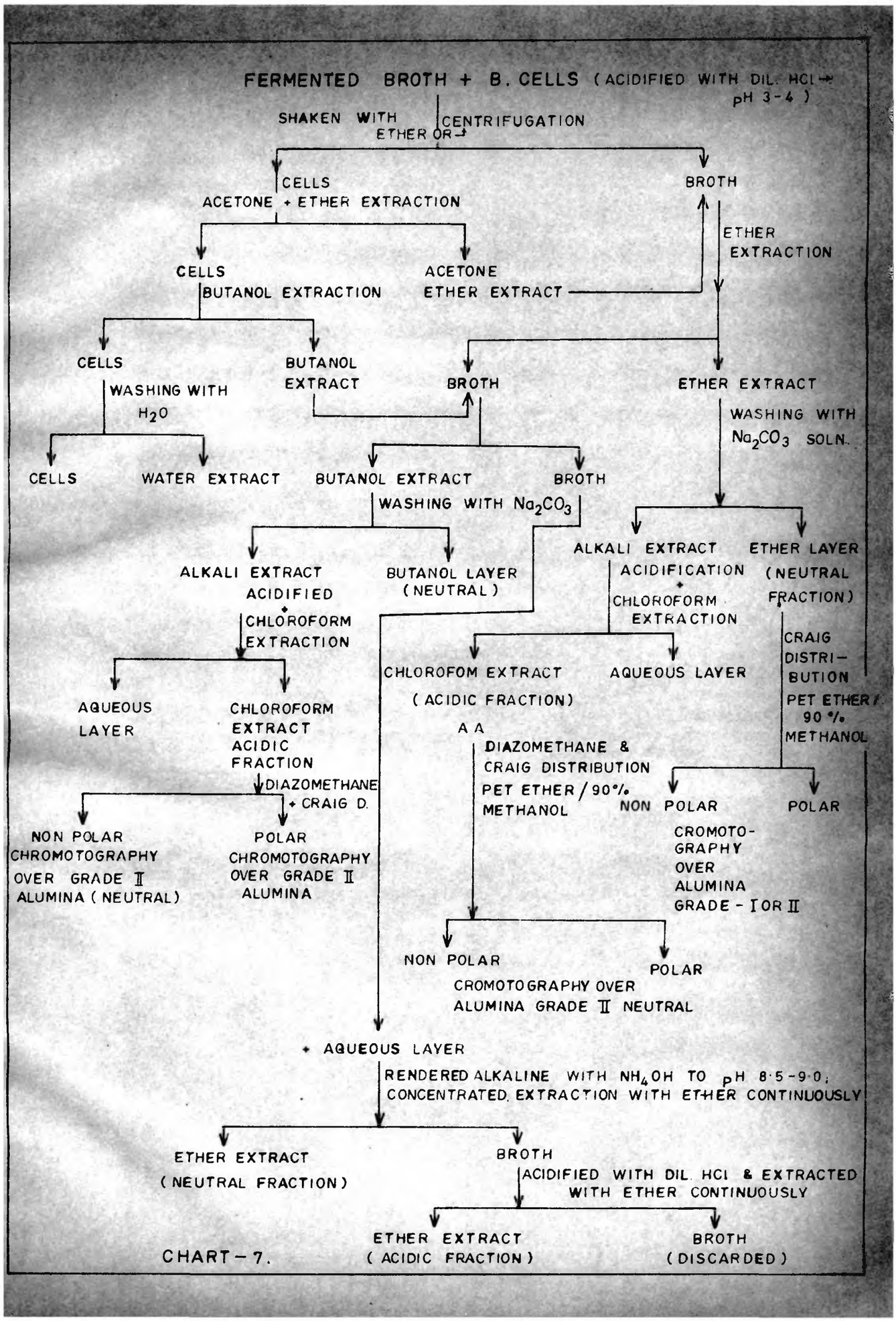
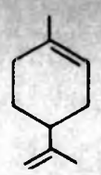

FERMENTED BROTH + B. CELLS (ACIDIFIED WITH DIL. HCl → pH 3-4)
SHAKEN WITH ETHER OR
CENTRIFUGATION
CELLS
ACETONE + ETHER EXTRACTION
BROTH
ETHER EXTRACTION
CELLS
BUTANOL EXTRACTION
ACETONE ETHER EXTRACT
CELLS
WASHING WITH H_2O
BUTANOL EXTRACT
BROTH
ETHER EXTRACT
WASHING WITH Na_2CO_3 SOLN.
CELLS
WATER EXTRACT
BUTANOL EXTRACT
BROTH
WASHING WITH Na_2CO_3
ALKALI EXTRACT
ACIDIFIED
+
CHLOROFORM EXTRACTION
BUTANOL LAYER (NEUTRAL)
ALKALI EXTRACT
ACIDIFICATION
+
CHLOROFORM EXTRACTION
ETHER LAYER (NEUTRAL FRACTION)
AQUEOUS LAYER
CHLOROFORM EXTRACT ACIDIC FRACTION
DIAZOMETHANE + CRAIG D.
CHLOROFOM EXTRACT (ACIDIC FRACTION)
AQUEOUS LAYER
CRAIG DISTRIBUTION PET ETHER / 90% METHANOL
A A
DIAZOMETHANE & CRAIG DISTRIBUTION PET ETHER / 90% METHANOL
NON POLAR
POLAR
NON POLAR CHROMOTOGRAPHY OVER GRADE II ALUMINA (NEUTRAL)
POLAR CHROMOTOGRAPHY OVER GRADE II ALUMINA
CROMOTOGRAPHY OVER ALUMINA GRADE - I OR II
NON POLAR
POLAR
CROMOTOGRAPHY OVER ALUMINA GRADE II NEUTRAL
+ AQUEOUS LAYER
RENDERED ALKALINE WITH NH_4OH TO pH 8·5-9·0; CONCENTRATED. EXTRACTION WITH ETHER CONTINUOUSLY
ETHER EXTRACT (NEUTRAL FRACTION)
BROTH
ACIDIFIED WITH DIL. HCl & EXTRACTED WITH ETHER CONTINUOUSLY
ETHER EXTRACT (ACIDIC FRACTION)
BROTH (DISCARDED)

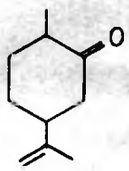
CHART - 7.

PRODUCTS OF BACTERIAL DEGRADATION OF LIMONENE

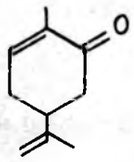


LIMONENE
(55)

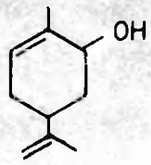
NEUTRAL PRODUCTS



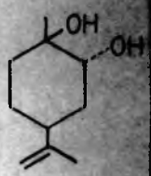
DIHYDROCARVONE
(56)



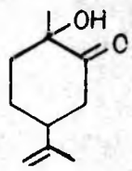
CARVONE
(57)



CARVEOL
(58)

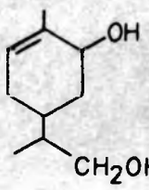


p- menth-8-ene
1,2-Diol
(62)



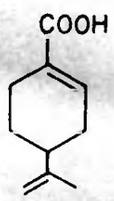
+ diol ?
3

p- menth-8-ene
1-ol-2-one
(60)

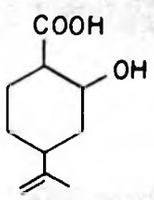


2,9-dihydroxy,
p-menth-1-ene
(64)

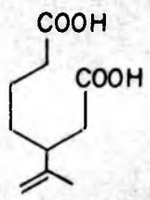
ACIDIC PRODUCTS



Perillic acid
(66)

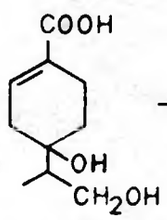


2-hydroxy
p-menth-8-ene
7-oic acid
(p-menth-8-ene
2-hydroxy,7-oic acid)
(77)



β-isopropenyl
Pimelic acid
(68)

+ Hydroxy acid (II)



4-9-dihydroxy
p-menth-1-ene 7-oic acid
(p-menth-1-ene 4-9 dihydroxy-7-oic acid)

→ DIHYDROXY ACID
?

CHART – 8

from ether and butanol solvents were washed with saturated brine, dried over sodium sulphate, concentrated and further separated into acidic and neutral components by washing with 5% sodium carbonate.

Neutral components

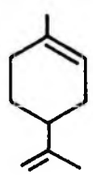
The neutral products after removal of ether were examined by thin layer chromatography (TLC). At least 8-10 components were noticed of which 5 were identified and characterized in the course of this investigation. This neutral fraction was subjected to a four transfer partitioning between light petroleum ether (N_1-I) and 90% aqueous methanol (N_1-II).

Petroleum ether layer (N_1-I) consisted mainly of unreacted limonene, some bacterial lipids and non-polar fractions which were then separated on alumina.

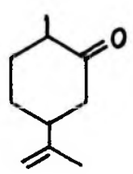
The aqueous methanolic layer (N_1-II) containing more polar hydroxylated transformation products were also subjected to chromatography over alumina.

Chromatography of petroleum ether layer (N_1-I) resulted in the separation of (i) unreacted limonene, bacterial lipids (Pet ether fraction); (ii) a ketonic fraction (Pet ether-ether fraction); (iii) a mono-hydroxylated compound (ether fraction) and (iv) traces of dihydroxy compounds (methanol fraction).

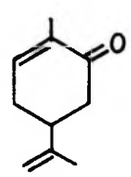
The ketonic fraction (N_1-I-ii) showed UV absorption at 235 mu characteristic of an α,β-unsaturated ketone but the ε value (10,000) fell somewhat short of what is expected for a pure compound. In thin layer chromatography it showed the existence of two spots. The Vapour-phase chromatograms also indicated two peaks. The n-m-r spectrum (Fig. 8) indicated that it contained a methyl group, α- to an α-β unsaturated carbonyl (8.34 τ), another methyl group on a disubstituted double bond of the type (8.27 τ), an isobutylenic double bond (5.27, 5.31 τ) and 5 protons situated on either side of an allylic carbon or on a carbon α- to a carbonyl system (7.6, 7.73 τ) and a vinyl proton at 3.42 τ. The n-m-r spectrum also revealed that the contaminant had a methyl group (split doublet at 9.10; 8.95 τ) which is adjacent to a carbon carrying a single proton. From these spectral data it was suspected that the ketonic fraction was constituted of a mixture of carvone (57) and dihydrocarvone (56), the former predominating.



(55)

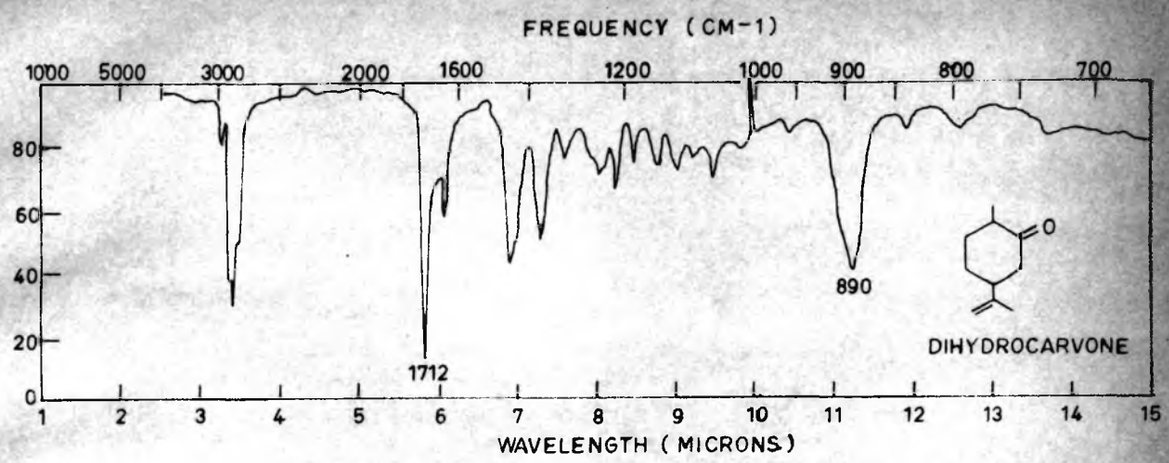


(56)



(57)

The principle used in an effective separation of these two ketones was that ketone (57) being an α,β-unsaturated


FREQUENCY (CM-1)
1000 5000 3000 2000 1600 1200 1000 900 800 700
80
60
40
20
0
1712
890
O
DIHYDROCARVONE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

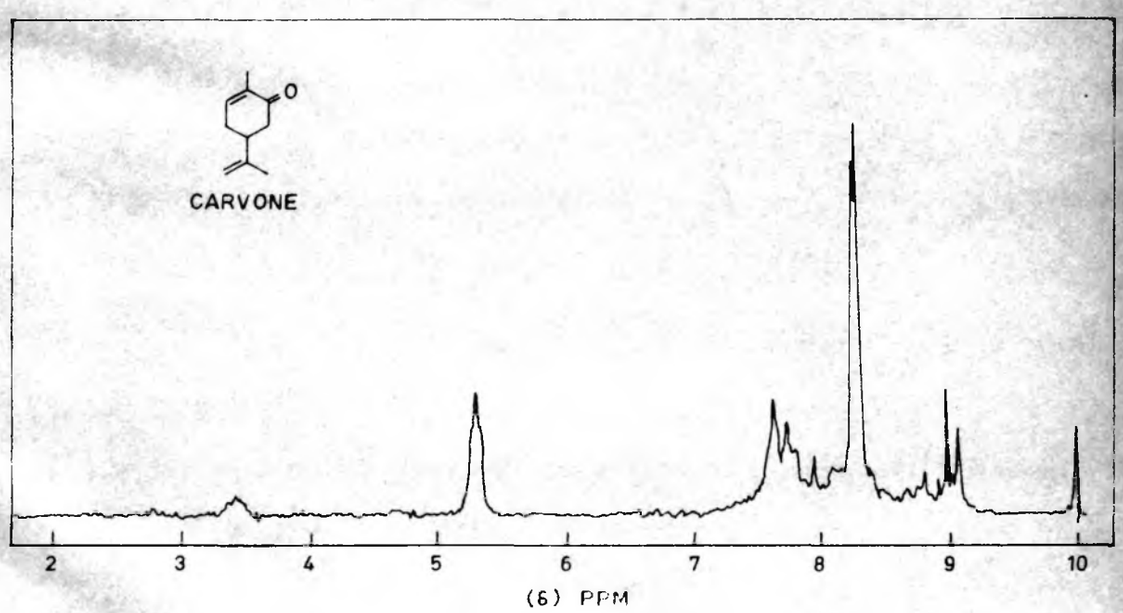

O
CARVONE
2 3 4 5 6 7 8 9 10
(δ) PPM

FIG.- 8.

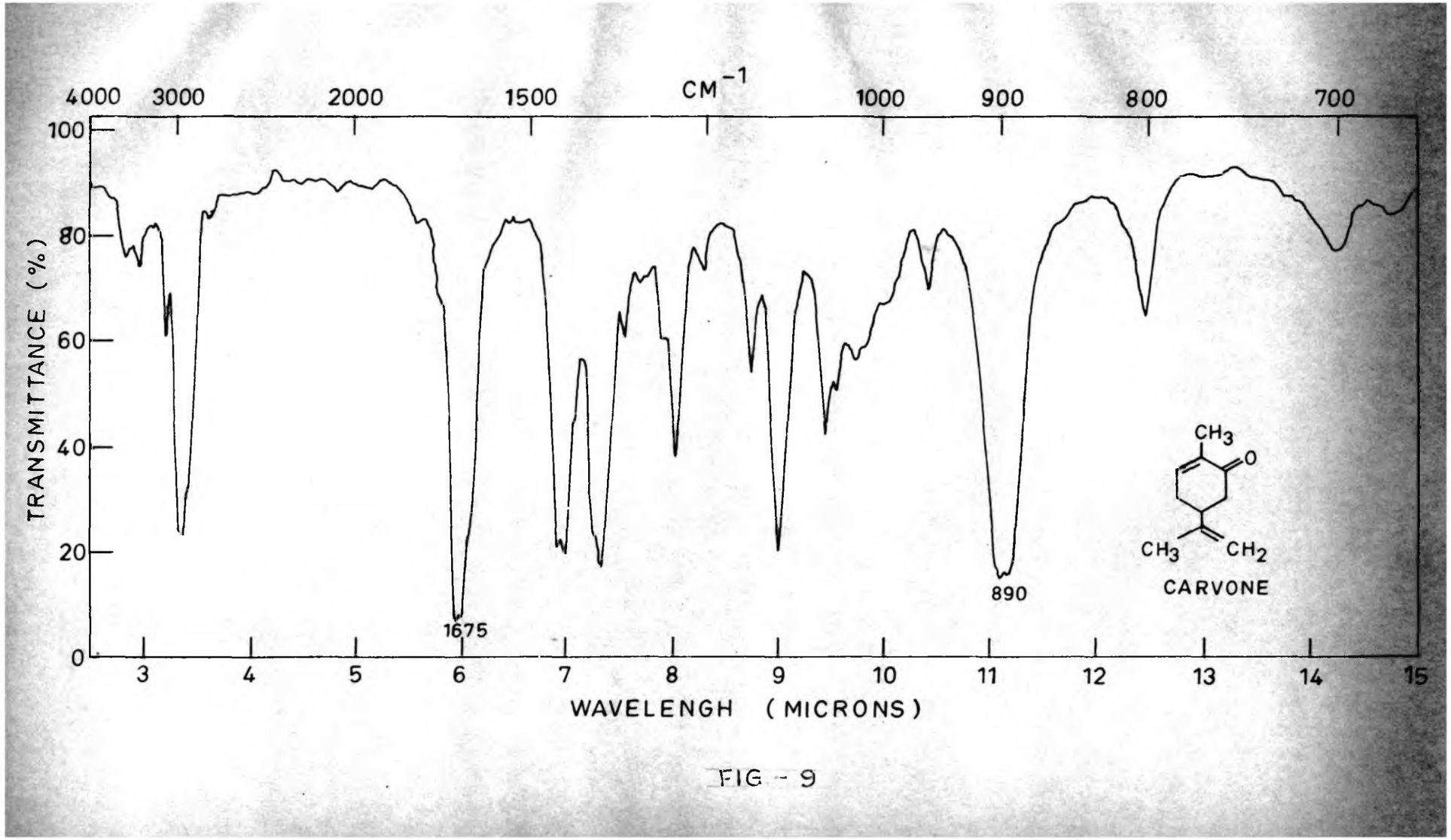

4000
3000
2000
1500
CM^{-1}
1000
900
800
700
100
80
60
40
20
0
TRANSMITTANCE (%)
1675
890
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGH (MICRONS)
CH_3
O
CH_3
CH_2
CARVONE

FIG - 9

carbonyl was expected to give a bisulphite adduct, whereas ketone (56) was not. The sulphite addition was accomplished under an effective control of pH by progressive addition of acetic acid to the disappearance of phenolphthalein colour. The unreacted ketone (57) was extracted with ether. The I.R. spectrum of this compound (Fig. 8) was identical with that of dihydrocarvone (bands at 1712, 1645, 890 cm^{-1}). The α,β-unsaturated ketone (57) was released from the bisulphite adduct by rendering the aqueous phase alkaline and recovered in ether. It was identified as a carvone (57) from its UV and I.R. spectra (Fig. 9) as well as through its 2,4-dinitrophenylhydrazone derivative. Furthermore, comparative thin layer chromatography, as well as vapour--liquid partition chromatography proved its identity with carvone.

The monohydroxy compound (N_1-I,iii) (58) eluted with ether showed a hydroxy band in the I.R. spectrum (Fig.10) (3355, 1080 cm^{-1}), an exocyclic methylene double bonds (1642, 888 cm^{-1}) and a trisubstituted double bond (812 cm^{-1}). Its analysis agreed well with the molecular formula, $C_{10}H_{16}O$. It gave a monoacetyl derivative. The n-m-r spectrum (Fig.11) of the compound (58) showed bands at 8.28 τ corresponding to a methyl group on an isobutylenic double bond, a complex signal of three proton intensity between 7.5-8.05 τ due to three allylic protons, a single proton split into a triplet (6.15 τ) corresponding to a single proton adjacent to a methylene group on a same carbon carrying a hydroxyl,

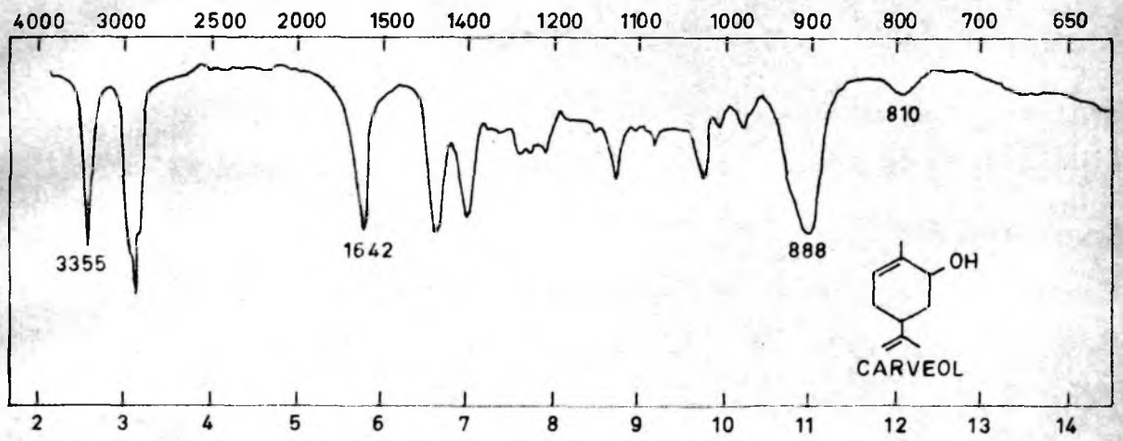

4000 3000 2500 2000 1500 1400 1200 1100 1000 900 800 700 650
3355
1642
888
810
OH
CARVEOL
2 3 4 5 6 7 8 9 10 11 12 13 14

FIG.- 10

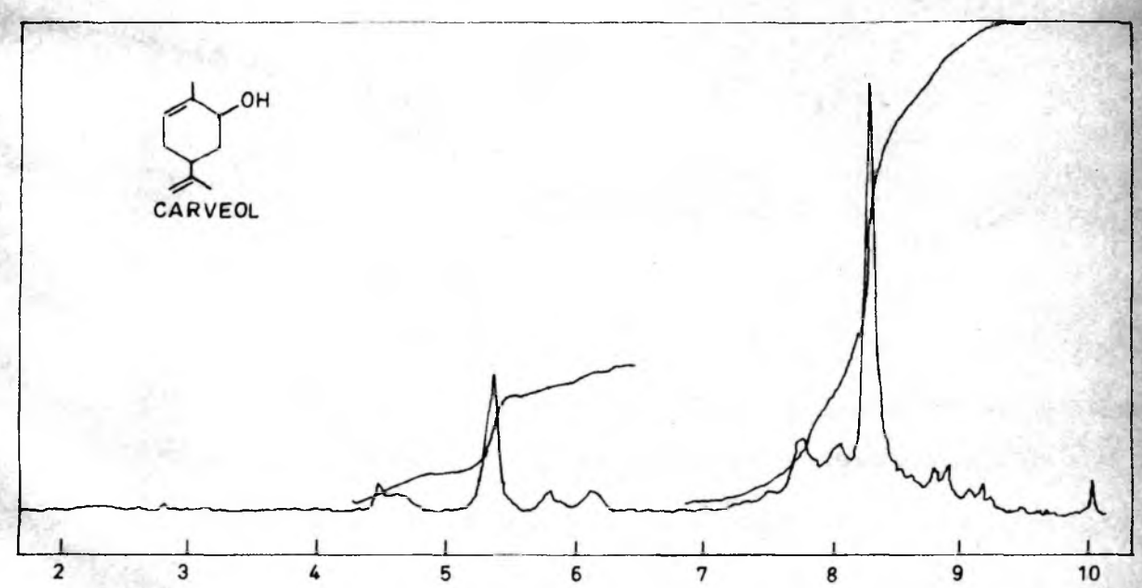
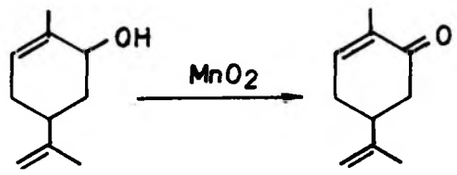

OH
CARVEOL
2 3 4 5 6 7 8 9 10

FIG - 11

an undifferentiated peak of two proton intensity at 5.35 τ assigned to the isobutylenic protons of the side chain, and a complex signal at 4.45 τ of a single proton intensity corresponding to vinyl proton split by two adjacent methylene protons. On manganese dioxide oxidation (Attenburrow, 1952) it gave carvone (57). These data allowed the monohydroxy derivative to be identified as carveol (58), and the specific rotation indicated its identity as d (+) carveol.



Separation of Polar Neutral Fraction

The fraction (N_1-I-iv) containing traces of liquid hydroxy compounds was pooled with the methanol layer (N_1-II). Methanol (90%) fraction (N_1-II) when chromatographed over grade II neutral alumina resulted in the following fractions :

(i) Traces of carvone, dihydrocarvone etc. (pet-ether fraction).

(ii) Diol. 1 (5% ether).

(iii) Hydroxy ketone (10%-50% ether).

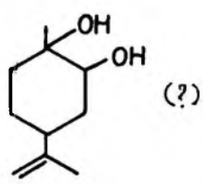
(iv) Diol. 2 (50% ether-100% ether).

(v) Diol. 3 (+ small quantity of diol 2) (100% ether).

(vi) Diol. 3 + more polar fractions. (methanol).

From the pet-ether (5%) fraction a small quantity of a liquid hydroxy compound - Diol 1 (59) (N_1-II-ii) with varying yields was obtained. This compound was associated

with carveol and the hydroxy ketone as trace impurities. Its separation and further purification were carried out by distillation under reduced pressure. The elementary analysis of this compound corresponded with a molecular formula $C_{10}H_{18}O_2$. The compound was periodate active and took up one mole of periodate. It gave a monoacetyl derivative indicating the presence of one primary or secondary hydroxy group. Its I.R. spectrum (not shown) showed a hydroxy group/groups. (νmax. 3355, 1056, 1112 cm^{-1}) and an exocyclic double bond (886 cm^{-1}). From the above data the Diol-1 is given the tentative structure (59) stereoisomeric with Diol-2. The assignment of <u>cis</u> configuration in Diol-1 is consistent with the reduced polarity. (Diol-2 showed identical behaviour towards acetylation and periodic acid treatment). However due to paucity of material no further work could be done on this compound.



(59)

The hydroxy ketone (N_1-II-iii) (60) eluting out with Pet-ether-ether fraction was inveriably associated with a small quantity of dihydroxy compound (62). Thin layer chromatography and the infrared spectrum indicated

the presence of a polar impurity in the sample. The separation of the hydroxy ketone (60) from the more polar dihydroxy compound was achieved by taking advantage of the insolubility of the dihydroxy compound (62) in carbon tetrachloride in the cold. The hydroxy ketone (60) being soluble in carbon tetrachloride was removed from the diol 2 by repeated extraction with carbon tetrachloride and cooling. The hydroxy ketone (60) so obtained was further purified by fractional distillation in vacuo.

The analysis agreed with the molecular formula $C_{10}H_{16}O_2$. The I.R. spectrum (Fig.12) indicated the presence of a tertiary hydroxy group (3450 and 1125 cm^{-1}) and a carbonyl band at 1700 cm^{-1} corresponding to a saturated cyclohexanon and an exocyclic methylenic double bond (887 cm^{-1}). The n-m-r spectrum (not shown) indicated the presence of a methyl on a tertiary carbon carrying an oxygen (signal at 8.82 τ) and an isobutylenic methyl (signal at 8.28 τ) and an isobutylenic methylenic group (signal at 5.3 τ). The structure of the hydroxy ketone (60) was established as follows.

The compound (60) took up one mole of periodate indicating that both the hydroxy and ketonic groups are adjacent to each other. The compound could not be acetylated with acetic anhydride and pyridine indicating that the hydroxyl group was possibly tertiary in nature.

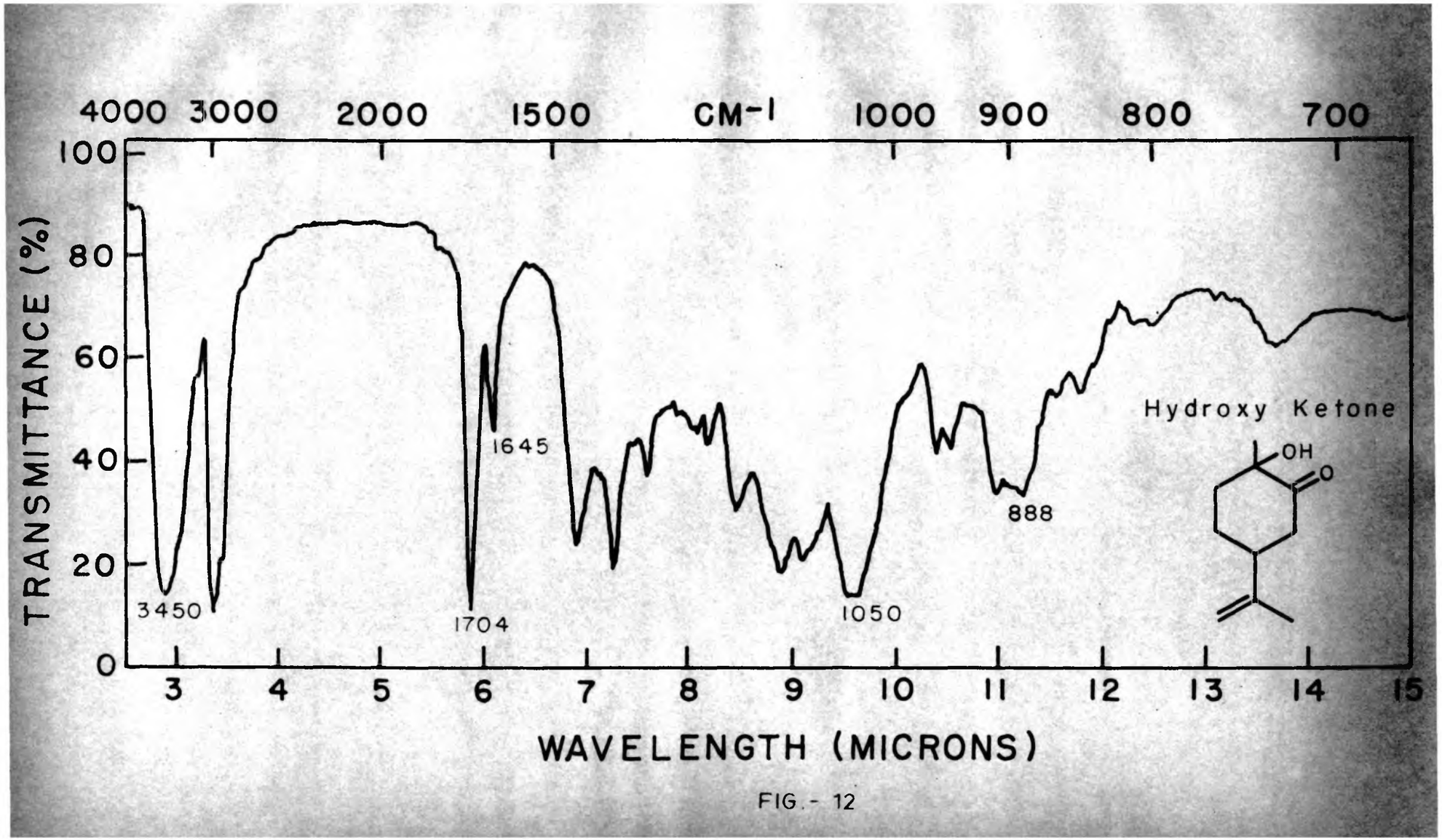
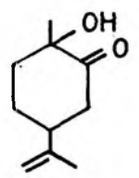
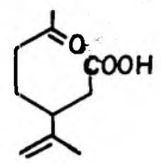

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
3450
1704
1645
1050
888
Hydroxy Ketone
OH
O
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG. - 12

The periodate cleavage product, (presumably the keto acid). (61),



(60)



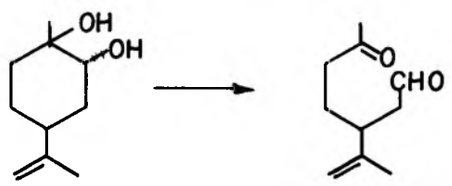
(61)

was extracted after acidification and was found to give an iodoform test. This established the structure of the hydroxy ketone as (60), with the absolute configuration not known.

The 50-100% ether fraction contained a dihydroxy compound (62) (N_1-II-iv) in substantial quantity. Thin layer chromatography showed that it was not a single compound but consisted of small impurities of the hydroxyketone and a more polar hydroxy compound. The separation of the diol was carried out as follows :

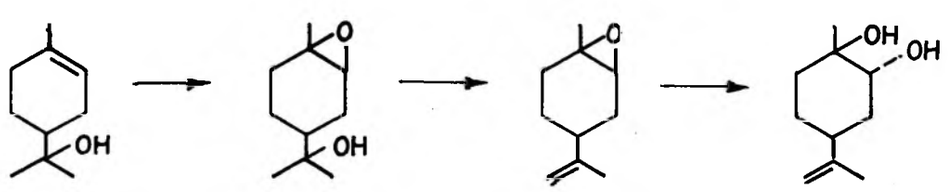
Preliminary experiments showed that when this dihydroxy compound (62) was dissolved in light petroleum and chilled, the addition of small quantity of carbon tetrachloride resulted in the separation of major hydroxy compound (62) leaving the hydroxy ketone (60) and the other dihydroxy compound (64) in solution. The crystalline material (m.p. 63-64^{o}) (62) obtained by the addition of carbon tetrachloride showed a strong hydroxy band in the

infrared spectrum (3215, 1160, 1115, 1048 cm^{-1}) (Fig. 13). It also showed the exocyclic-isobutylenic double bond (890 cm^{-1}). The elemental analysis of the compound (62) corresponded with the molecular formula $C_{10}H_{18}O_2$. In the UV it showed only an end absorption at 209 mμ , ε 3600 indicating that the double bond is unsymmetrically disubstituted. The compound took up one mole of periodate. It yielded a neutral keto-aldehyde (63) responding to the iodoform reaction.



(62) (63)

The compound (62) yielded a mono acetate (I.R. νmax 3590, 1750, 1250, 1042, 922 cm^{-1}). These data are in full accord with the assigned structure (62) for the diol 2. This diol appears to have the same properties as that of the p-menth-8-ene-1,2-trans diol obtained by the oxidation of α-terpineol (Schmidt 1949; Newhall 1964).



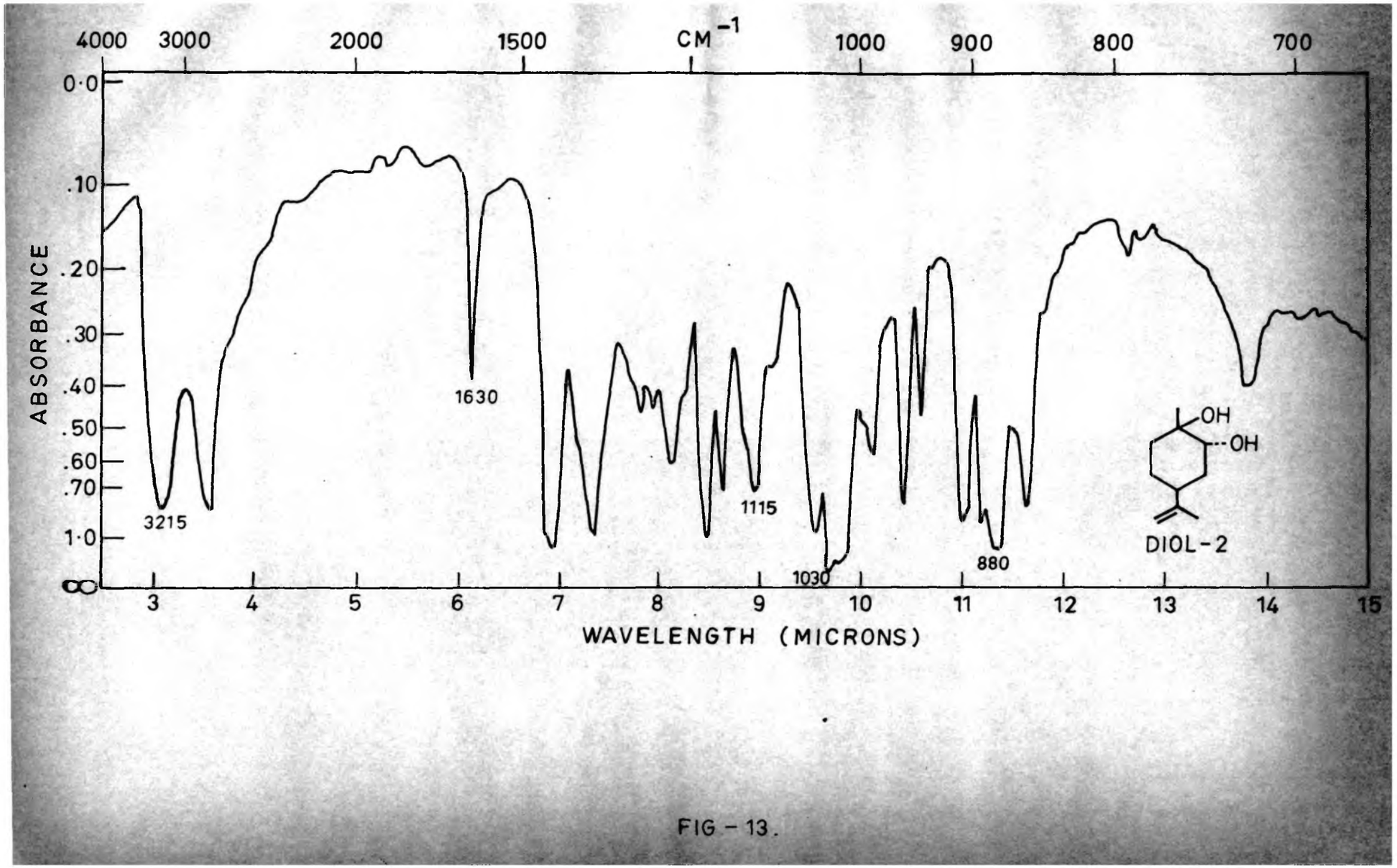
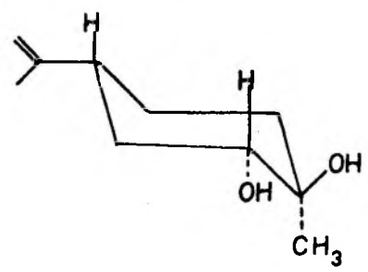

4000
3000
2000
1500
CM -1
1000
900
800
700
0·0
.10
.20
.30
.40
.50
.60
.70
1·0
∞
ABSORBANCE
3215
1630
1115
1030
880
OH
OH
DIOL-2
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

FIG - 13.

The n-m-r spectrum (Fig.14) indicated the presence of a methyl on a tertiary carbon carrying oxygen (8.85 τ) and an isobutylenic methyl (8.32 τ) and a methylene group (5.37 τ). The single proton on the carbon carrying the other oxygen function appeared at 6.56 τ and showed spin coupling with the protons of an adjacent methylene group. These data are consistent with the structure (62a) for the diol-2.

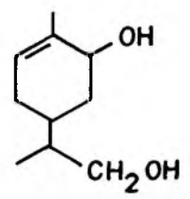


(62a)

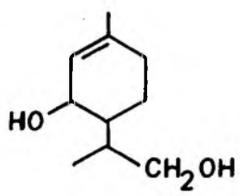
The fraction coming out from 100% ether and methanol contains a mixture of highly polar dihydroxy alcohols (N_1-II-vi). Thin layer chromatography revealed the existance of two spots of which one was the major ($\sim$ 80%) component. The separation of these alcohols was carried out by rechromatography (N_1-II-vi) on alumina. The fraction eluting with 100% chloroform (64) was found to be chromatographically pure. It analysed for $C_{10}H_{18}O_2$. The infrared spectrum (Fig. 15) indicated the presence of hydroxy groups (3400, 1048, 1016 cm^{-1}). This compound was inert towards periodate action. It gave a diacetyl derivative (disappearance of 3400 cm^{-1} band) indicating that both the hydroxy groups are in a non-hindered position.

The n-m-r spectrum (Fig.14) indicated the presence of a methyl group on a saturated carbon containing a proton (split signal at 8.96 τ , J = 4.5 cps), a methyl group on a trisubstituted double bond (8.37 τ), a vinyl proton (4.65 τ) showing spin coupling with two adjacent methylene protons, two protons of a carbon carrying a primary hydroxyl (6.38 τ) and a single proton on a carbon carrying secondary hydroxyl (6.6 τ) adjacent to a methylene group.

From the n-m-r spectrum (Fig. 14) and the other physico chemical evidences the tentative structure[64] could be assigned to Diol 3.



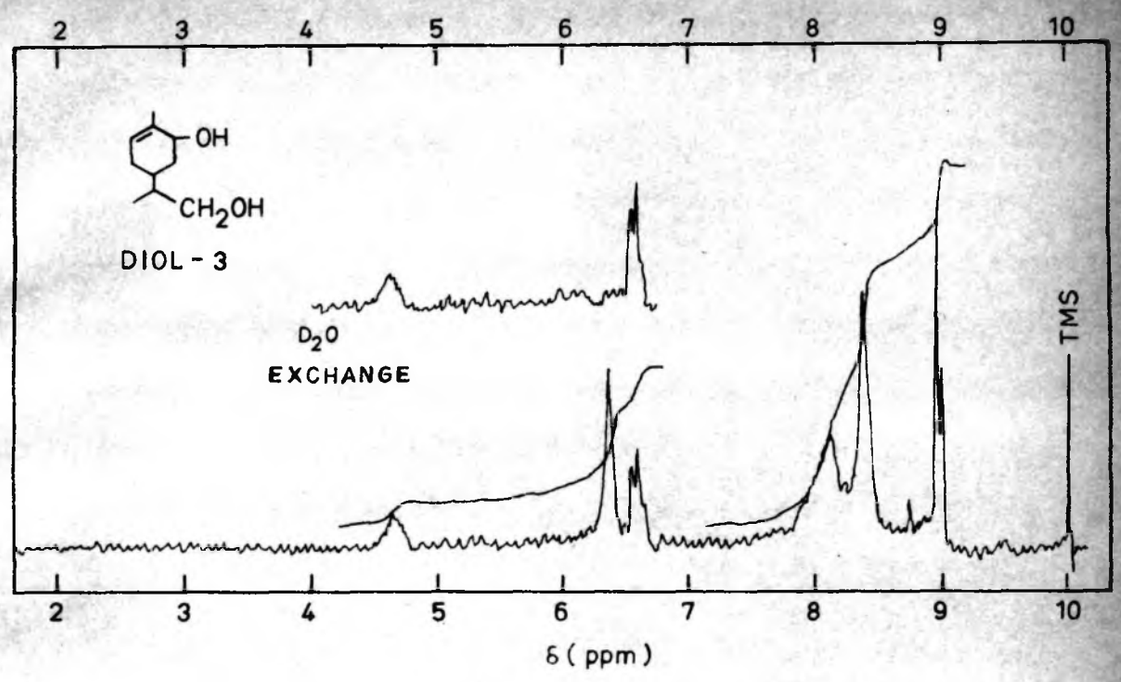
(64)



(64a)

It should be noted that the assignment of structure 64 satisfies the observation that the proton on the carbon carrying the hydroxyl appears as a triplet 6.6 τ . This behaviour rules out the alternative structure 64a.

Further evidence in support of the structure 64 was obtained by the oxidation of the compound 64 with manganese dioxide to the α-β unsaturated ketone (65) with an absorption at 235-236 mμ . This indicated that the


2 3 4 5 6 7 8 9 10
OH
CH_2OH
DIOL - 3
D_2O
EXCHANGE
TMS
2 3 4 5 6 7 8 9 10
δ (ppm)

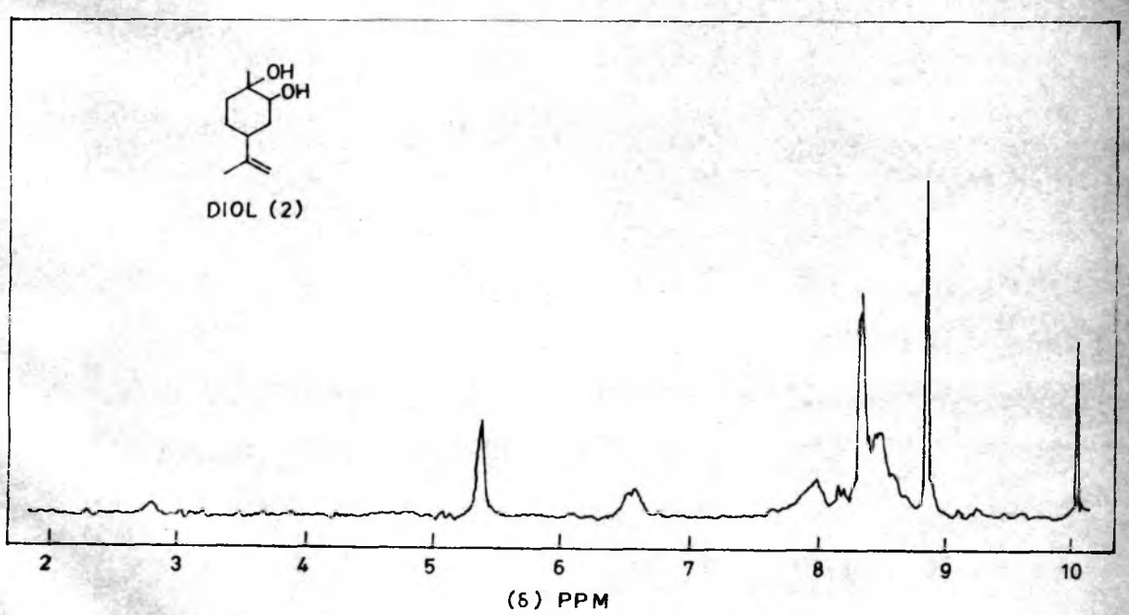

OH
OH
DIOL (2)
2 3 4 5 6 7 8 9 10
(δ) PPM

FIG.- 14 .

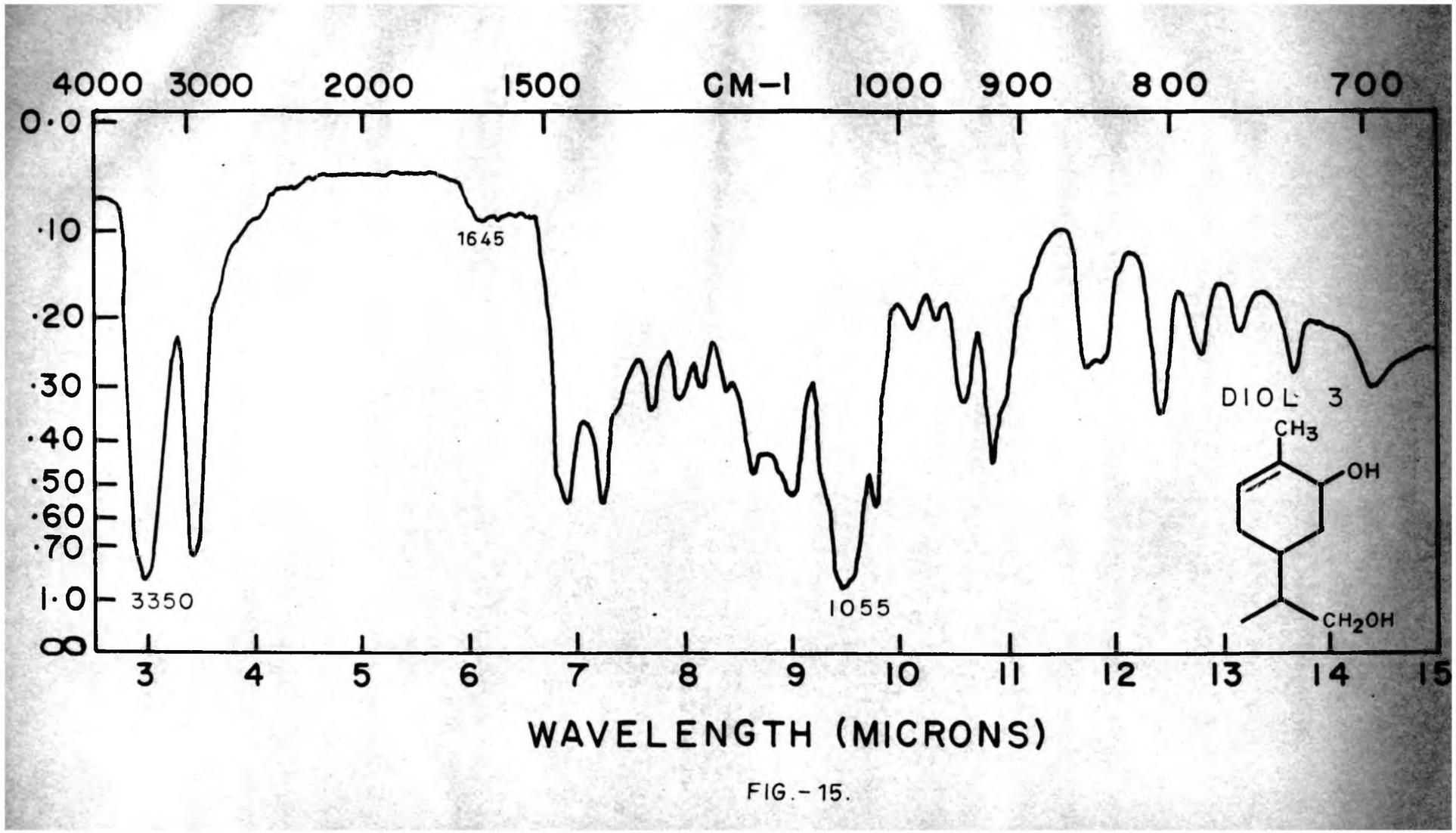
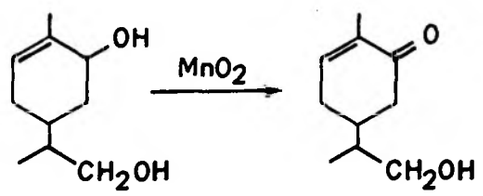

4000 3000 2000 1500 CM-1 1000 900 800 700
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
∞
1645
3350
1055
DIOL 3
CH3
OH
CH2OH
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG.-15.

secondary hydroxyl group in 64 is located in an allylic position. However, the structure 64 remains to be rigorously proven.



(64) (65)

It was not possible to obtain the remaining products from the mixture in quantitiessufficient for the elucidation of their structures.

Acidic components

The acidic components from the sodium carbonate extract when subjected to thin layer chromatography indicated at least four major spots in the approximate proportions of ~ 25:50:15 and 5 and a few minor spots. Three of the major acidic components from ether and butanol extracts were identified, while the fourth acid could not be fully identified and characterised due to its unstable nature and the paucity of material.

The acidic fractions (A_1) were converted into their methyl esters (A_1Me) with diazomethane and subjected to a four transfer partitioning between light petroleum ether and 90% aq. methanol. The extracts from the petroleum ether (A_1Me-I) and methanol (A_1Me-II) were concentrated separately

and subjected to chromatography separately over alumina (30 times the wt. of the material). The following fractions were collected :

(i) Fatty acid ester (pet ether).

(ii) Monohydroxy ester (2-10% ether).

(iii) Perillic acid methyl ester (10-25% ether).

(iv) Traces of a dicarboxylic acid (50-100% ether).

The first fraction (A_1Me-I-i) eluted with pet-ether was a semisolid m.p. 42-45^{o} which was identified as fatty ester from spectral studies and analysis. The second fraction eluted from 2-10% ether (A_1-Me-I-ii) when chromatographed on TLC showed a single spot but over VPC it exhibited two peaks (40:60). Cyclohexyl amine salt of one of the acids was obtained m.p. 110 (I.R. bands 1700, 1618 cm^{-1}) (not shown). The elementary analysis of the liberated acid indicated a molecular formula $C_{10}H_{16}O_2$. The I.R. spectrum indicated that the compound had a trisubstituted double bond. However the quantities of the acid obtained were not sufficient for further work.

The third fraction (A_1 Me-I-iii) eluting from column with ether (10-25%) showed a single spot on TLC. The elementary analysis of the ester (67) corresponded to the molecular formula, $C_{11}H_{16}O_2$, in accordance with a methyl ester of a monocarboxylic acid. The U.V. spectrum indicated the presence of an α,β-unsaturated ester

(λ max, 218 mμ ; ϵ , 8107). The infrared spectrum (Fig.17) of this compound showed a sharp ester band at 1710 cm^{-1} (α,β-unsaturated ester band), 1250 cm^{-1}, and an exocyclic methylenic double bond (1645 and 890 cm^{-1} bands) and a trisubstituted double bond (780 cm^{-1}). The shoulder at 1615 cm^{-1} is suggestive of the α-β-unsaturation in the molecule.

After saponication of this ester (67) the corresponding acid was obtained as a colourless crystalline compound (66) which after recrystallization from pet-ether melted at 124-125°. (literature, Herz and Wahlberg, 1962, m.p. of perillic acid 129° - recrystallised from ethanol). The n-m-r spectrum (Fig. 18) of the acid indicated the presence of an isobutylenic system (methyl protons at 8.24 τ and the methylenic protons at 5.27 τ), five allylic protons (appearing as a broad signal around 7.75 τ) and a vinyl proton at the β-position on the α,β-unsaturated system (2.86 τ). From the spectral data it was indicated that the acid may be perillic acid (66).

A spectral comparison (Fig. 16) of the bacterial metabolite and an authentic sample of perillic acid revealed that the compounds were identical. There was no depression in the mixed m.p. The methyl ester of both acids i.e. that of the bacterial metabolite and of an authentic sample showed the same retention time over vapour phase chromatogram.

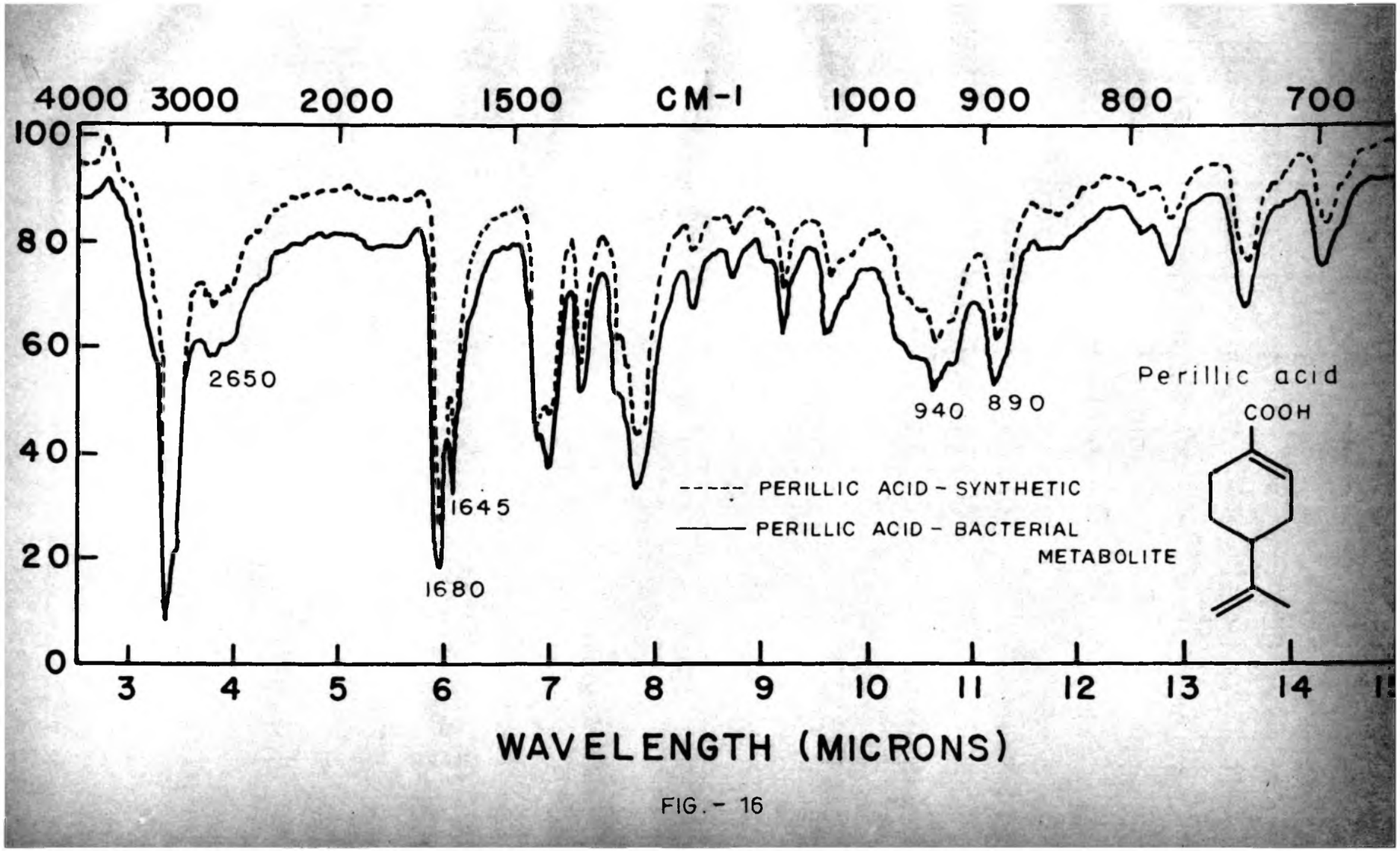

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
2650
1645
1680
940
890
Perillic acid
COOH
PERILLIC ACID - SYNTHETIC
PERILLIC ACID - BACTERIAL METABOLITE
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

FIG. - 16

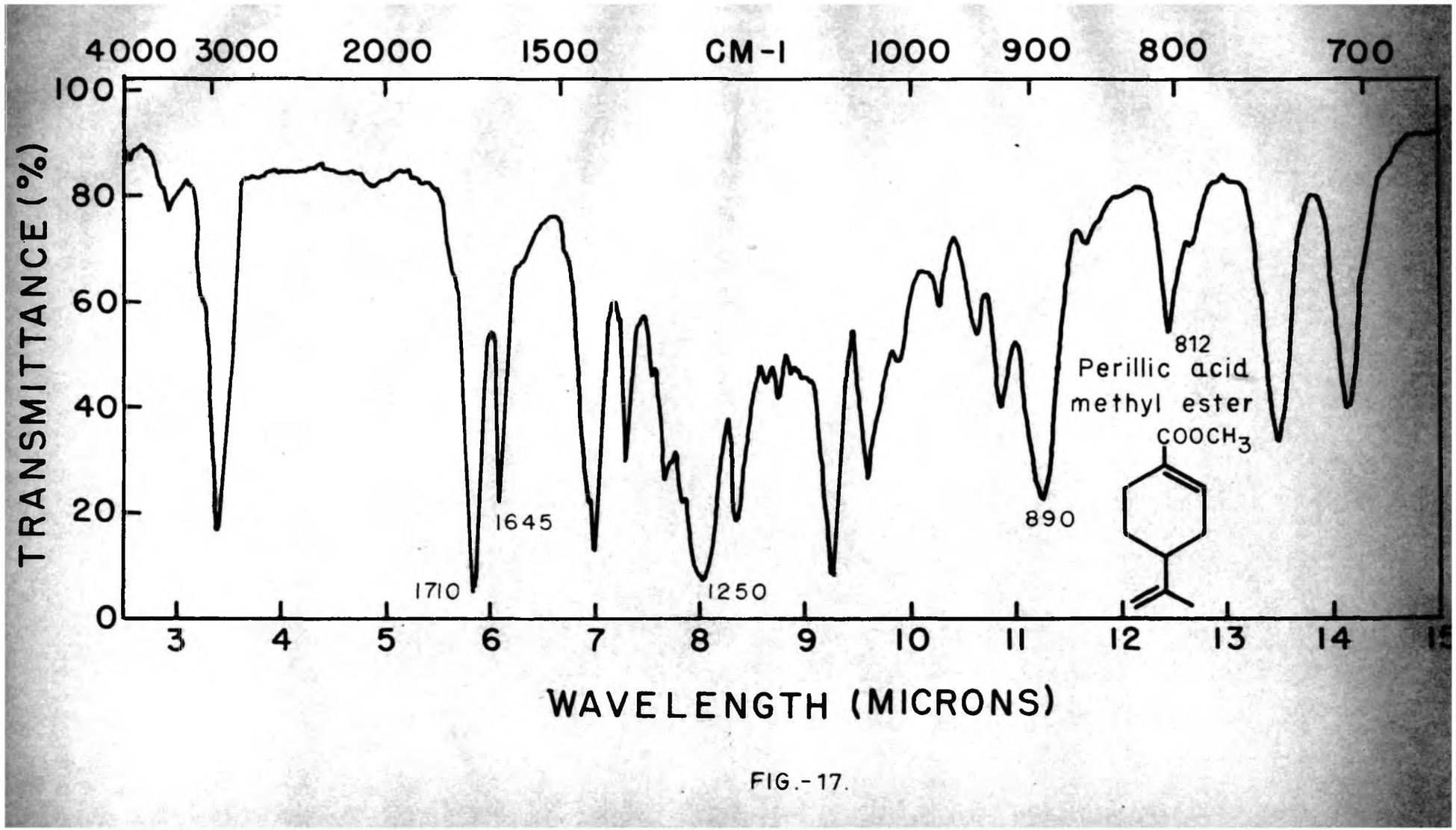
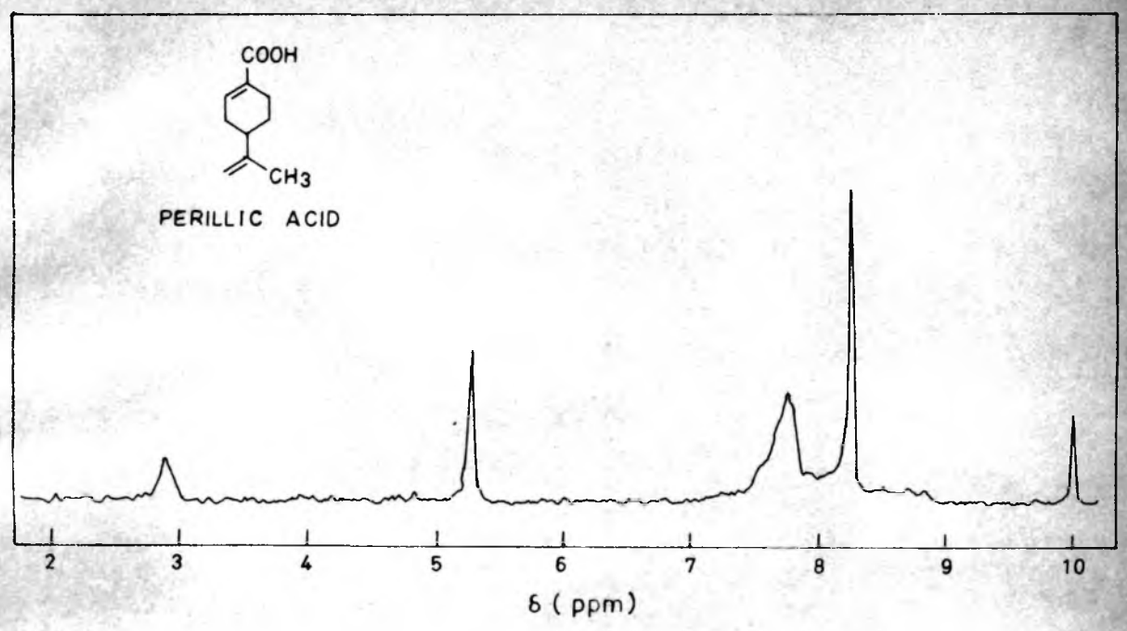

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
1710
1645
1250
890
812
Perillic acid
methyl ester
COOCH3
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

FIG.-17.


COOH
CH3
PERILLIC ACID
2
3
4
5
6
7
8
9
10
δ (ppm)

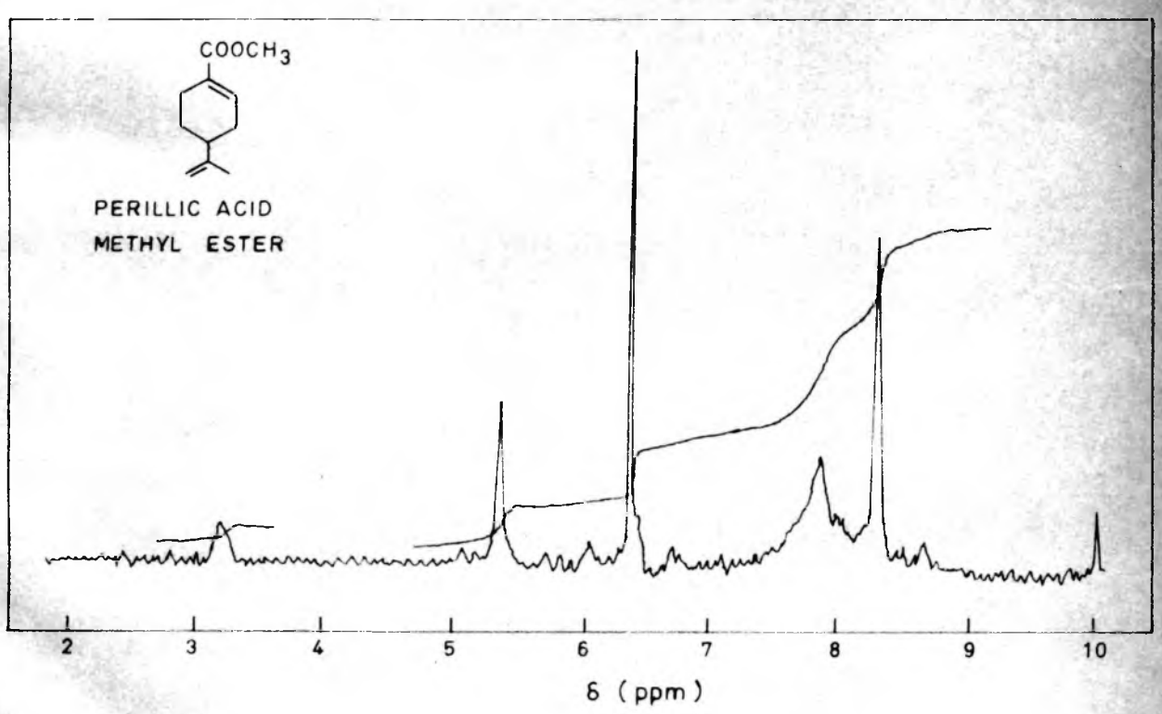
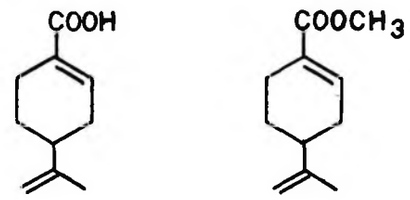

COOCH3
PERILLIC ACID
METHYL ESTER
2
3
4
5
6
7
8
9
10
δ (ppm)

FIG. - 18.



(66) (67)

Fraction (A_1 Me-I-iv) eluted with 50-100% ether gave traces (very small quantity) of a polar compound which was identified as the dicarboxylic acid (68).

No other acids could be isolated in large amounts from the petroleum ether fraction (A_1 Me-I) by column chromatography.

Chromatography of the methyl esters from the methanol fraction (A_1Me-II)

The methyl esters from the 90% methanol phase were chromatographed over alumina and eluted with the usual solvents. First pet ether fraction eluted a liquid compound which was identified through its I.R. and analysed to be a fatty acid ester. The second fraction eluted with 2-5% ether was a liquid monocarboxylic ester which after distillation under reduced pressure and chromatography over TLC and VPC was identified as perillic acid methyl ester (67). Pet ether-ether (10-50% ether) eluted a dimethyl ester (A_1Me-II-iii) in substantial amounts. Vapour phase chromatograms and TLC indicated that this fraction (69) was almost pure. The elemental analysis and molecular

weight determination of compound (69) indicated the molecular formula $C_{12}H_{20}O_4$. It did not show any characteristic UV bands besides an end absorption (λ max. 208-210 mμ). The I.R. spectrum (Fig. 19) displayed an ester band (1740, 1250 cm^{-1}) corresponding to a saturated carboxylic acid as well as an isobutylenic methylenic absorption (900 cm^{-1}). It was evident from the finger print region of the infrared spectrum (Fig. 19) that there was no carbocyclic ring in this compound. Both the acid and the ester were optically inactive. The n-m-r spectrum (Fig. 20) of the ester (69) indicated the presence of an isobutylenic system (methyl proton signal at 8.33 τ and methylenic protons at 5.28 τ), two carbomethoxy methyls at (6.33 τ) and 5 protons either allylic to double bonds or α- to a carbonyl system (complex signals at 7.7 τ to 7.9 τ). On saponification of the diester the free acid (68) was obtained as a thick viscous liquid which showed characteristic bands for carboxyl groups (Fig. 21) ( )max. 2650, 1708, 940 cm^{-1}) and the presence of an exocyclic methylenic double bond (898 cm^{-1}).

The di-cyclohexylamine salt of the dicarboxylic acid was obtained as colourless crystalline solid by treating the acid with cyclohexylamine in acetone (m.p. 148-150°). The I.R. spectrum (Fig. 22) indicated typical carboxylate salt bands (2280, 1645 cm^{-1}). The n-m-r spectrum of the free acid (Fig. 20) essentially corresponded that of the dimethyl esters, excepting for the bands for the ester methyls

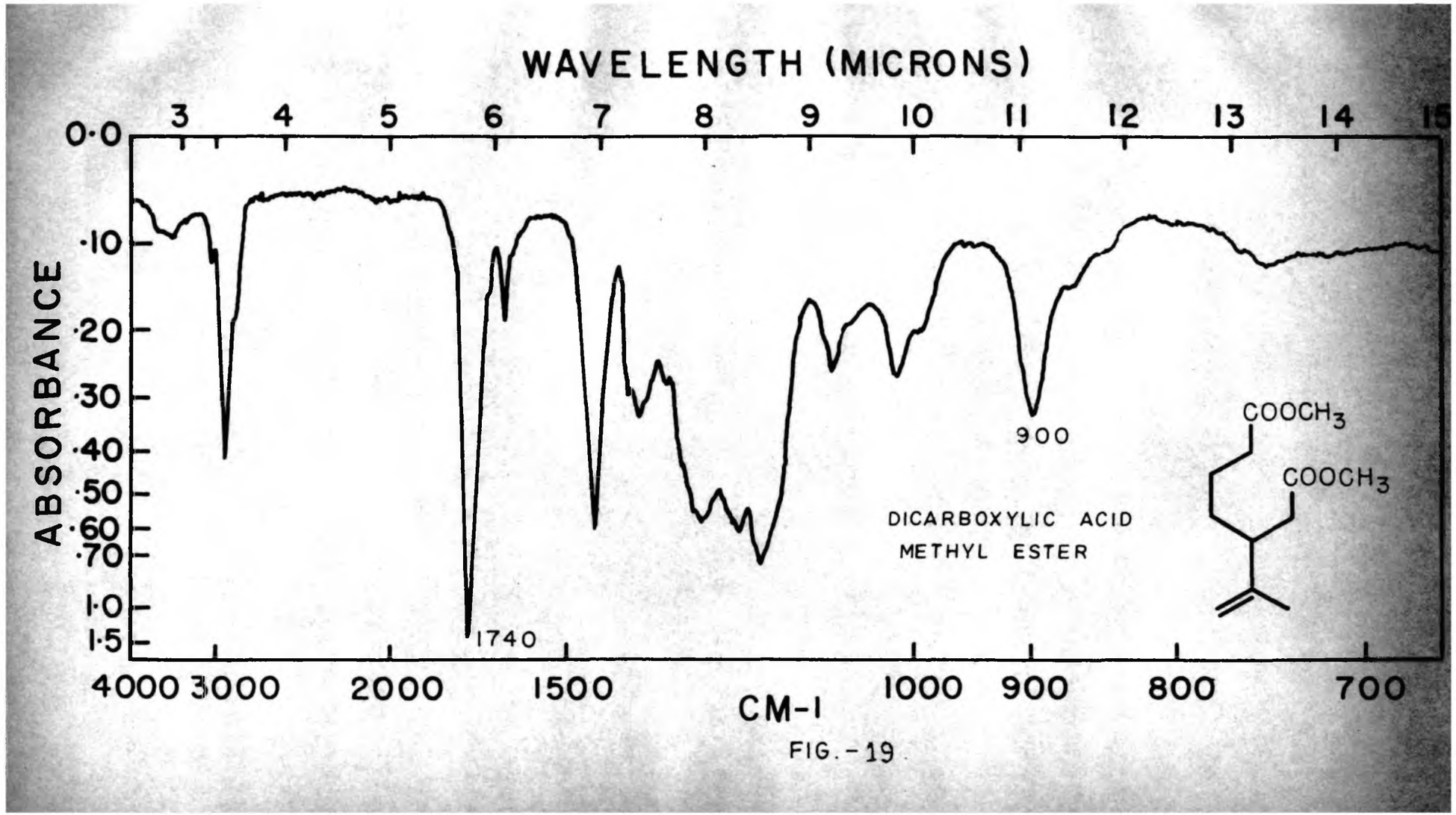
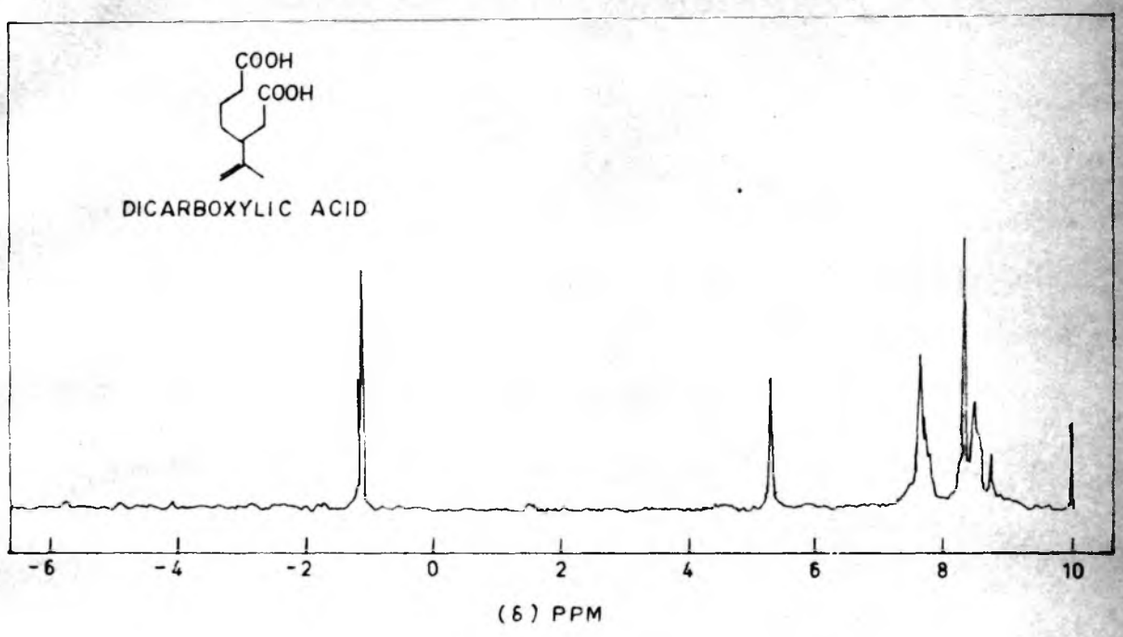

WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
11
12
13
14
15
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
1·5
ABSORBANCE
1740
900
COOCH3
COOCH3
DICARBOXYLIC ACID
METHYL ESTER
4000
3000
2000
1500
1000
900
800
700
CM-1

FIG.-19


COOH
COOH
DICARBOXYLIC ACID
-6
-4
-2
0
2
4
6
8
10
(δ) PPM

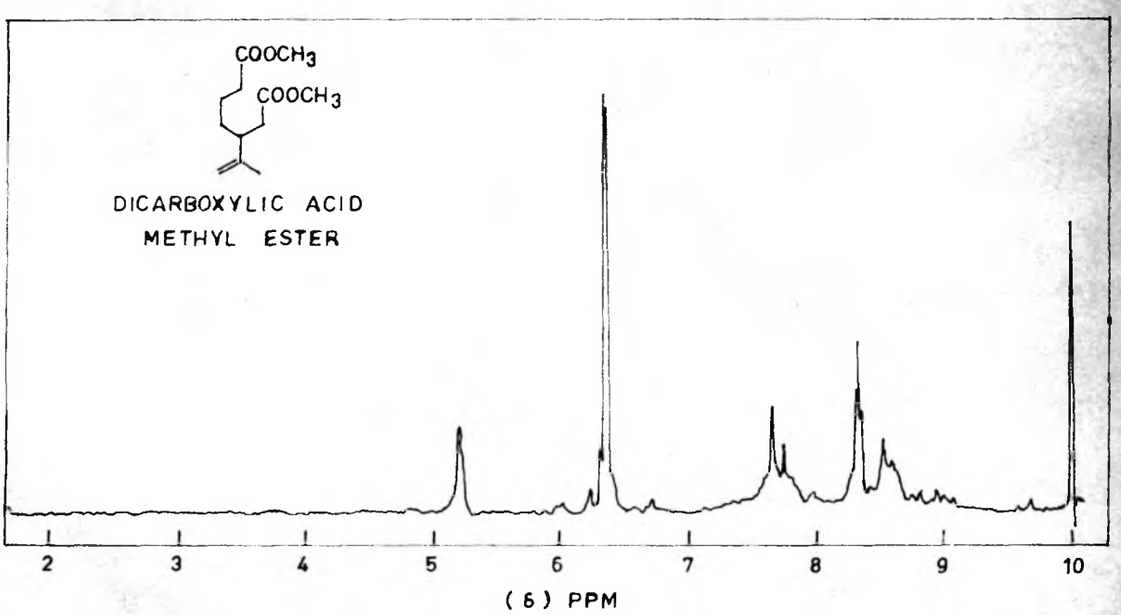

COOCH3
COOCH3
DICARBOXYLIC ACID
METHYL ESTER
2
3
4
5
6
7
8
9
10
(δ) PPM

FIG.-20.

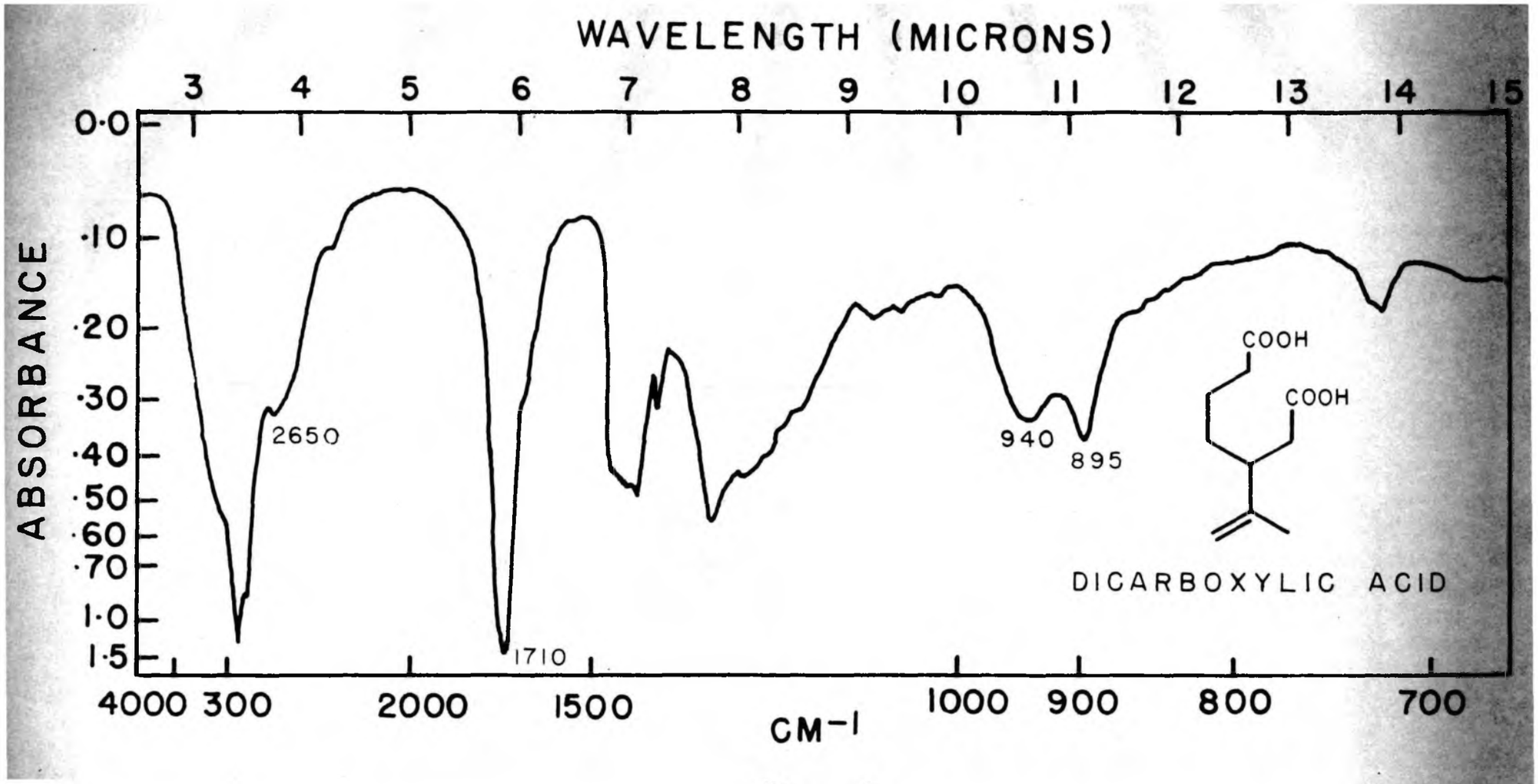

WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
11
12
13
14
15
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
1·5
ABSORBANCE
2650
1710
940
895
COOH
COOH
DICARBOXYLIC ACID
4000
300
2000
1500
1000
900
800
700
CM-1

FIG.– 21.

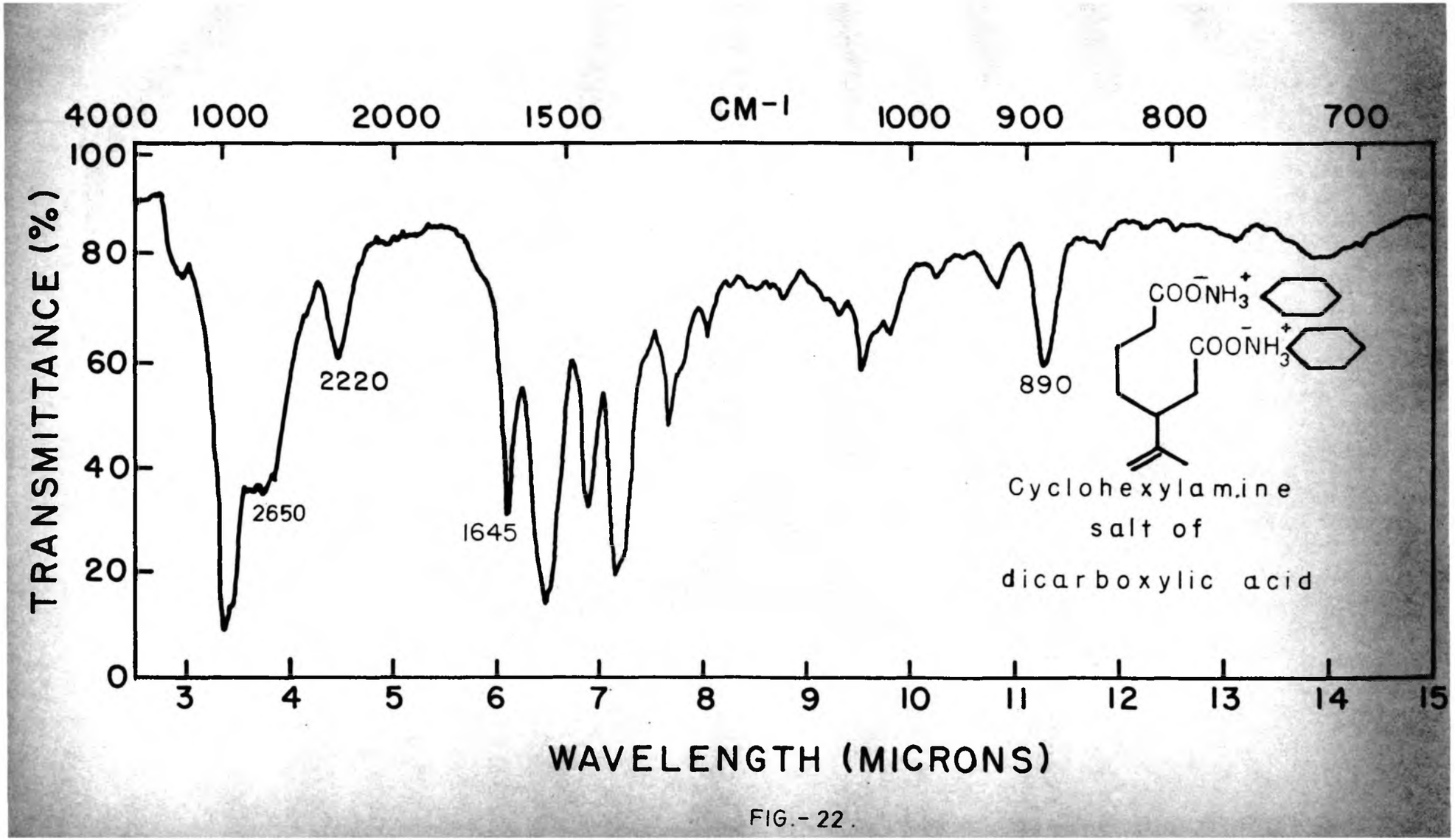
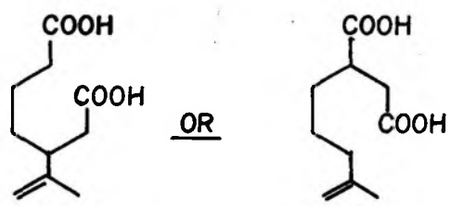

4000 1000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
2650
2220
1645
890
COO⁻NH₃⁺
COO⁻NH₃⁺
Cyclohexylamine
salt of
dicarboxylic acid
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG.- 22.

From the above physical data two possible structures (68) and (68a) can be assigned to the dicarboxylic acid. However with structure (68a) it is difficult to see how the double bond would be stable in the isobutylenic position.



(68) (68a)

It was possible to distinguish between the structures 68 and 68a by heating the acid with acetic anhydride. Compound 68a was expected to yield a substituted succinic anhydride which would show characteristic anhydride bands at 1860 and 1780 cm^{-1}. (Bellamy, 1957a). However, only after prolonged heating, a product was isolated which showed bands at 1815, 1770, 1730 cm^{-1} corresponding to a mixed anhydride.

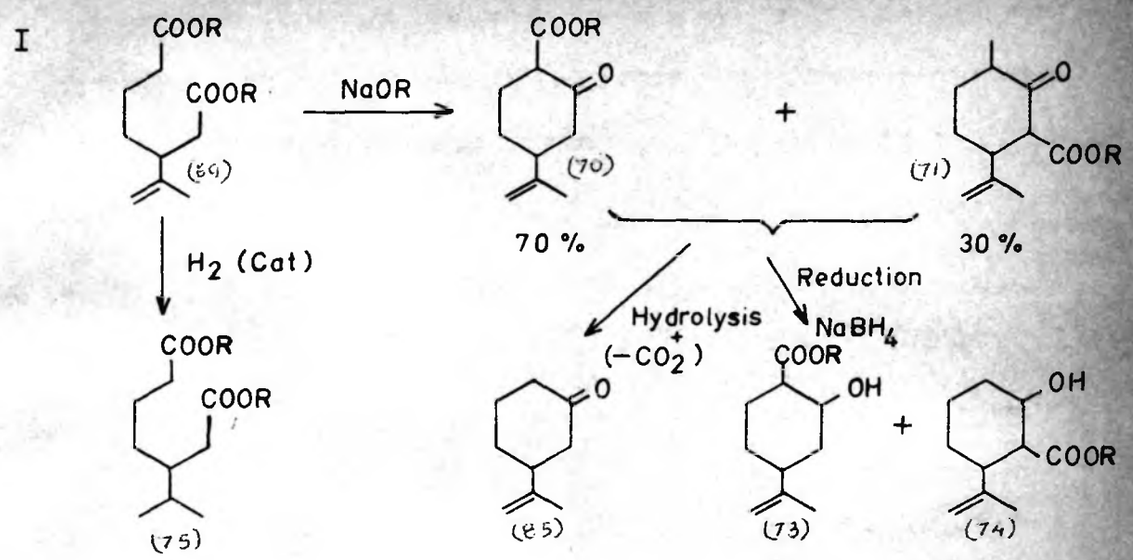
In order to establish the structure more definitely, a Dieckmann's cyclisation reaction was carried out with dimethyl ester of the acid (69). The product was found to contain a mixture of two isomeric keto esters (presumably 70 and 71) by vapour phase chromatography in the relative proportions of ~ 70 and 30. The product showed bands at 1750 and 1675 cm^{-1} characteristic of β-keto esters where the carbonyl group is on a six-membered ring. The Dieckmann's

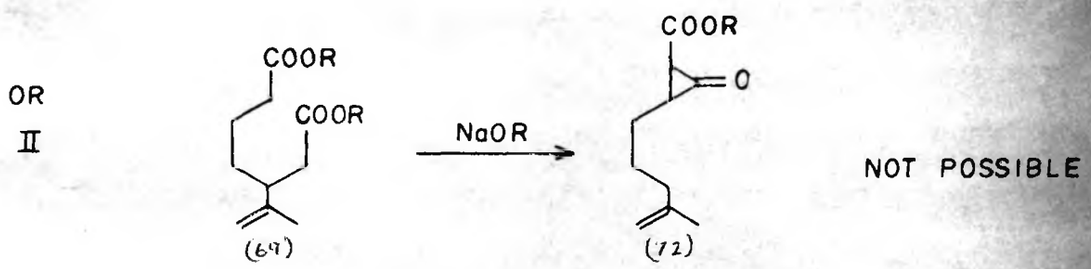
cyclization product for structure (68a) is expected to be a cyclopropane carboxylate (72) which would show carbonyl bands at much higher frequencies (Bellamy, 1957b).

The mixture of keto-esters 70 and 71 was reduced with sodium borohydride to a mixture of at least two hydroxy esters (presumably 73 and 74), one of which had identical mobility on a thin layer chromatogram and retention time on VPC column with that of a hydroxy acid isolated from the fermentation mixture (vide spectra). (Fig. 24).

When the dimethyl ester (69) was fully hydrogenated in the presence of platinum catalyst in alcohol one mole of hydrogen was taken up and a saturated diester (75) (disappearance of 890 cm^{-1} band) was obtained. The fully saturated diester (75) was identical with the diester obtained by Bhattacharyya et al (1964) in the fermentation of α,β-pinenes by a pseudomonad. Further support was obtained for the structure (68) by ozonolysis. Formaldehyde was obtained as a product of ozonolysis in 60-70% yield and was characterized as its dimedone derivative (m.p. 186-187°, Lit. reported 189°, Vogel, 1956). When the ozonolysed product was worked up and the neutral fraction containing the keto-carboxylic ester (76) and the unreacted diester (40:60) (69) were chromatographed over alumina it yielded the keto ester (76) which was eluted with 75-100% ether. In the I.R. spectrum it showed an ester and a

STRUCTURE OF DICARBOXYLIC ACID





OZONOLYSIS OF DICARBOXYLIC ACID

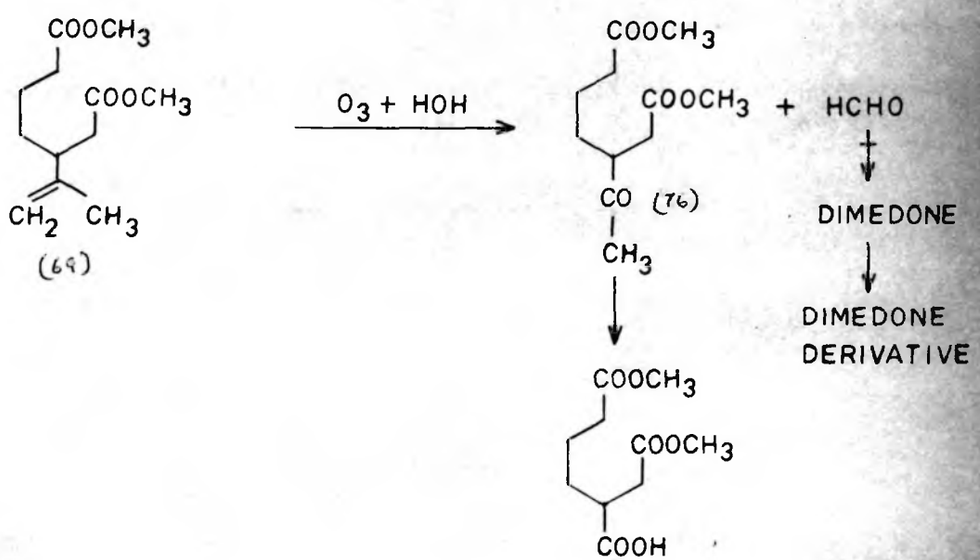


CHART – 9

ketone band (1732, 1710 cm^{-1}) with the disappearance of 890 cm^{-1} band. The keto ester gave iodoform test indicating that it was a methyl ketone.

After the dicarboxylic acid ester, the fraction (A_1 Me-II-iv) eluting out on 75-100% ether was a liquid which showed two spots on TLC. Even with 100% ether and 100% chloroform small amount of this acid was obtained contaminated with the more polar hydroxy acid. In order to have effective separation, the column was eluted with 100% ether, then chloroform and finally with methanol. The combined polar esters were rechromatographed on alumina (A_1Me-II-iv) and eluted with the same solvent systems. The fraction eluting with 25-50% ether gave a liquid compound (73) (A_1Me-II-iv-a) with a slight impurity of a more polar hydroxy ester. The compound was further purified by distillation under reduced pressure.

The ester (73) analysed for $C_{11}H_{18}O_3$. . The I.R. spectrum (Fig. 24) displayed a hydroxy band ( )max. 3600, 1040 cm^{-1}) indicating presence of a secondary alcohol an ester band (1750, 1158 cm^{-1}) and bands at)max. 1645 and 892 cm^{-1} indicating isobutylenic type double band.

One of the hydroxy ester (73) obtained by reduction of the keto-esters 70 and 71 obtained in Dieckmann's cyclisation of the diesters showed identical mobility in TLC and retention time in the VPC with the above ester (73).

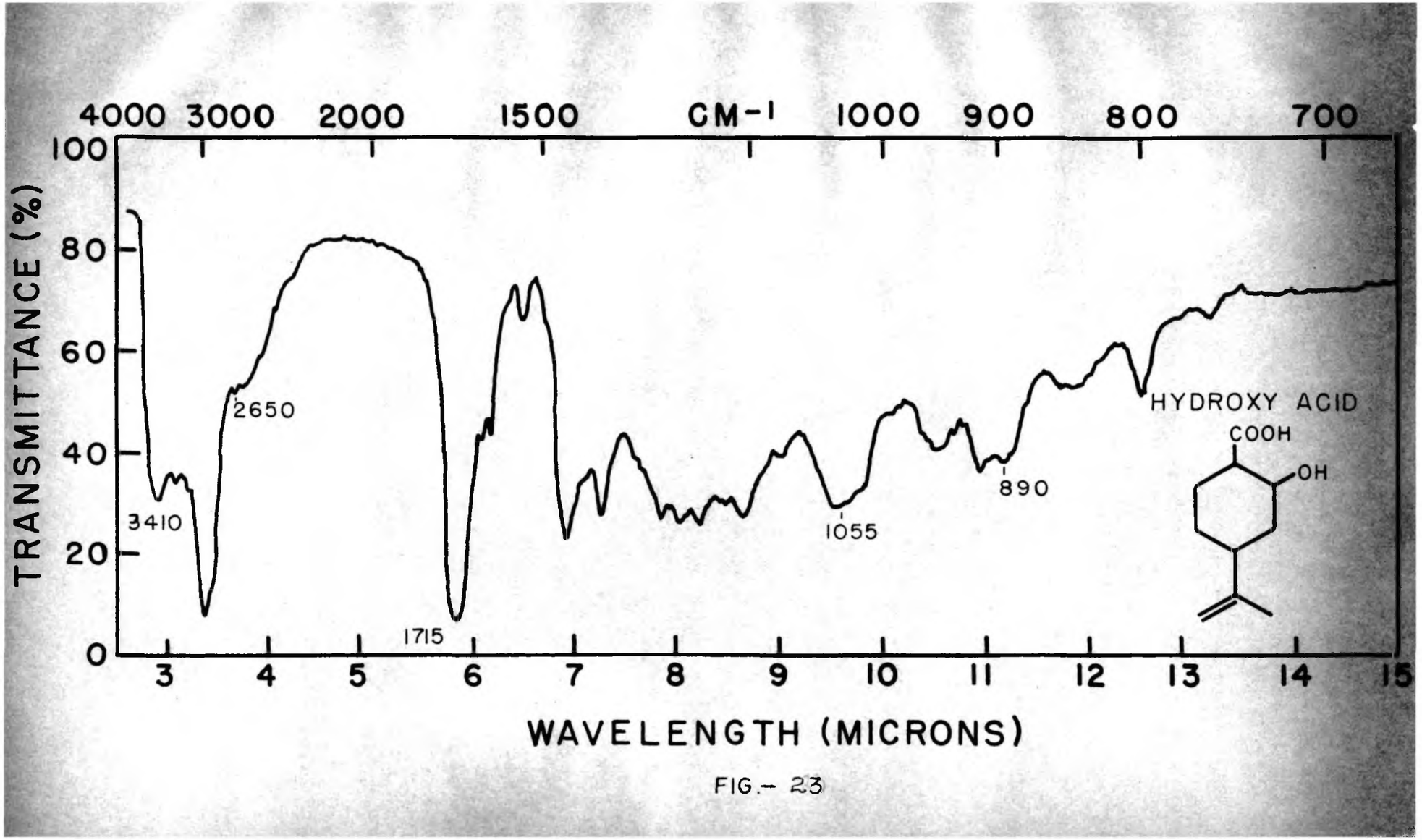

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
3410
2650
1715
1055
890
HYDROXY ACID
COOH
OH
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG.- 23

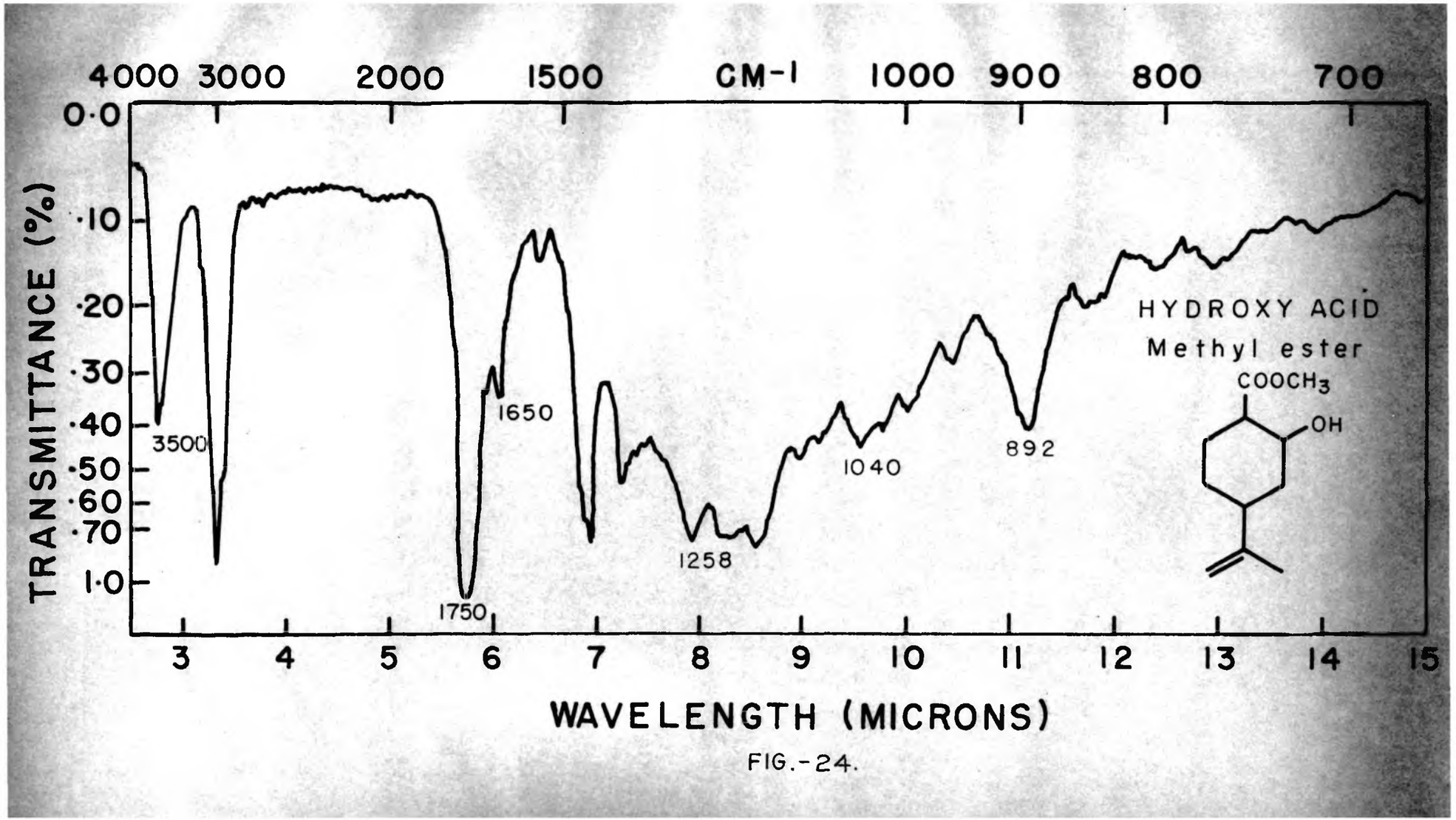
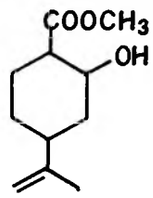
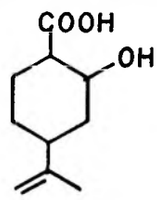

4000 3000 2000 1500 CM-1 1000 900 800 700
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
TRANSMITTANCE (%)
3500
1750
1650
1258
1040
892
HYDROXY ACID
Methyl ester
COOCH3
OH
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG.-24.

The n-m-r spectrum of the ester (Fig. 25) showed the presence of an isobutylenic system [methyl protons at 8.32 τ and methylene protons at 5.35-5.43 τ (as a quadruplate)], a carbomethoxy methyl group and the proton on a carbon carrying a secondary hydroxyl group as a complex signal on four protons intensity at (6.32-6.38 τ) and one proton α to a carbonyl and an allylic proton both around 7.63, 7.7 τ coupled with an adjacent methylene groups. These spectral data were consistent with structure 73 for the ester.



(73)



(77)

On saponification compound (73) gave the free hydroxy acid (77) as a viscous liquid. The I.R. spectrum (Fig. 26) of this compound showed absorption due to the hydroxyl group (3410, 1050 cm^{-1}), a carboxy group (2650, 1715 cm^{-1}) and a isobutylene double bond (1645, 890 cm^{-1}). The acid gave a monoacetyl derivative (I.R. ν_{max} 1730 cm^{-1}, disappearance of hydroxyl absorption). On chromic acid oxidation it gave a keto carboxylic acid (78) which when hydrolysed with mild alkali gave the original di-carboxylic acid (68). This series of transformations (~~Chart 9~~) adequately established the structure of the hydroxy acid (77) with the stereochemistry at position 1 and 2 uncertain.

The next fraction (A_1Me-II-iv-b) eluting out from the column with 50-100% ether was a yellow and slightly viscous liquid (TLC R_F 0.3) which was purified by repeated distillation in vacuo to give a colourless liquid. This fraction analysed for the molecular formula $C_{11}H_{18}O_3$. It showed a characteristic UV absorption (λ max. 218 mμ ; ϵ, 11,700) indicating an α,β-unsaturated ester group in the molecule. The I.R. spectrum of this ester (Fig. 26) showed a hydroxyl group (I.R. νmax. 3410, 1090, 1042 cm^{-1}), an α,β-unsaturated ester group (1708 cm^{-1}) and an exocyclic methylenic double bond (1650, 1615 shoulder - 905, 810 cm^{-1}). As the compound was unstable and available in small quantities, no further work was possible on this hydroxy ester.

Butanol extract of the broth fermentation yielded a small quantity of acidic fraction (A_2-I) which was identified as the dicarboxylic acid (68) by TLC, VPC and actual isolation of the acid as the hexylamine salt and spectral comparisons.

Apart from the four acidic compounds found in the normal extraction of the broth with ether and butanol, a crystalline acidic product was obtained in the continuous ether extract (A_3). This acidic fraction (79) was crystallised out from ether or chloroform (m.p. 180-182°).

The elementary analysis of the acid (79) led to the molecular formula $C_{10}H_{16}O_4$. The compound (79)

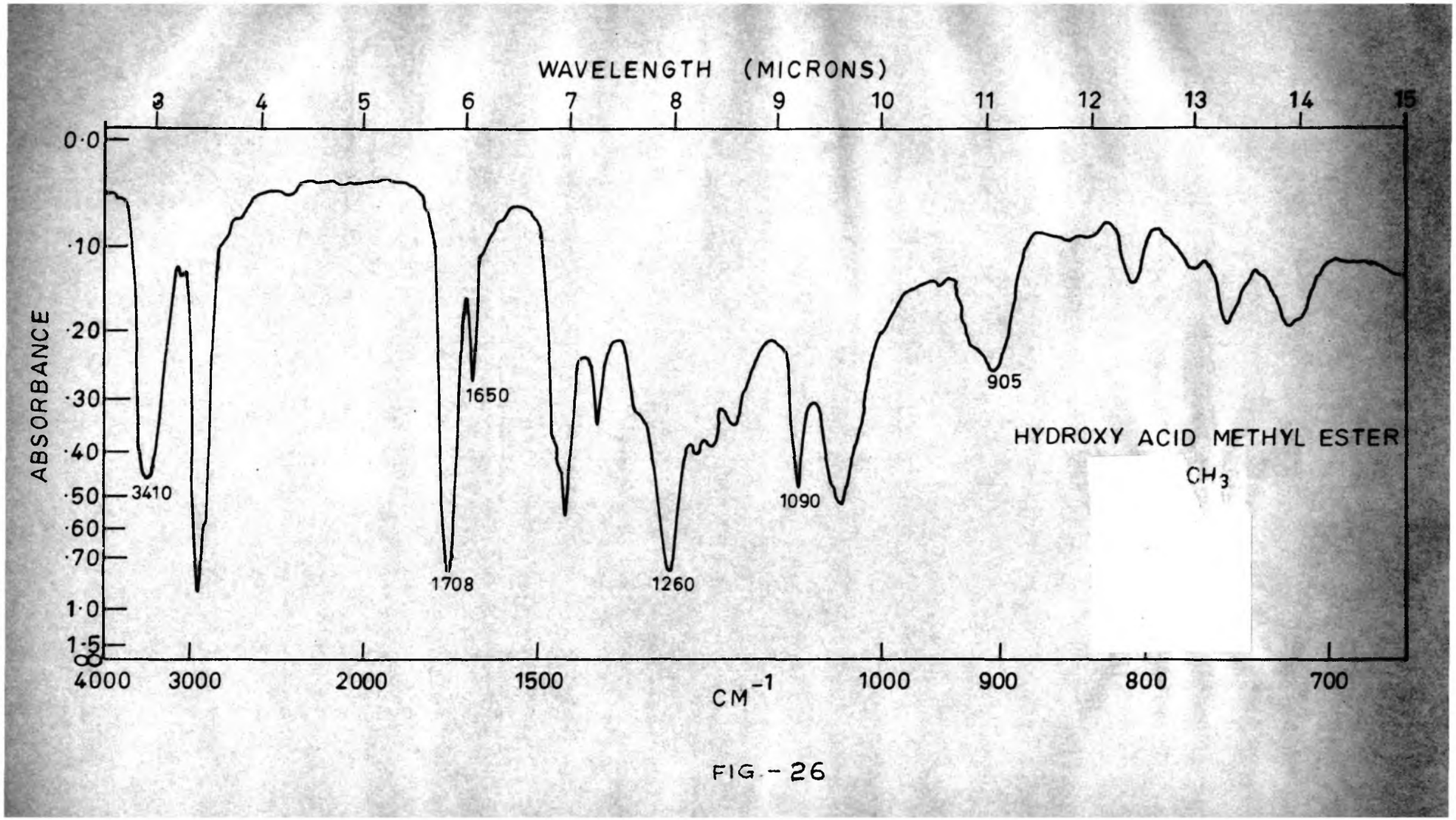

WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
11
12
13
14
15
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
1·5
ABSORBANCE
3410
1708
1650
1260
1090
905
HYDROXY ACID METHYL ESTER
CH3
4000
3000
2000
1500
1000
900
800
700
CM-1

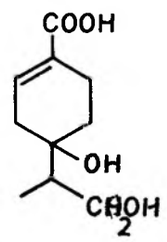
FIG - 26

showed UV absorption at λ max. 216 mμ, (ϵ, 12,800), indicating α,β-unsaturation in the system. The infrared spectrum (Fig. 29) indicated sharp bands for the hydroxy groups (3410, 1160, 1040 cm^{-1}) presumably due to the presence of a tertiary and a secondary hydroxyl group in the molecule. Bands at 2650, 1680 and 1655 cm^{-1} also supported the presence of an α,β-unsaturated carboxylic acid grouping in the molecule.

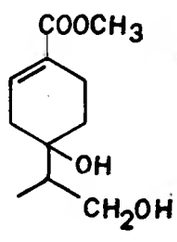
On esterification with diazomethane a solid methyl ester (80) was obtained (m.p. 110°, bp 210-220°, bath 1 mm). The infrared spectrum (not shown) supported the structure 80 for the ester (I.R. νmax. 3400, 1700, 1645 cm^{-1}).

On acetylation with acetic anhydride and pyridine one of the hydroxy group was acetylated indicating the other hydroxy group was possibly a tertiary one. (I.R. νmax. 3400, 1738 cm^{-1}).

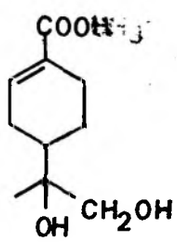
From the above data one of the two structures 80 and 80b are possible for the methyl ester.



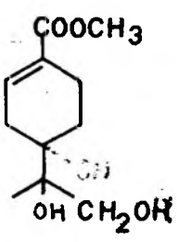
(79)



(80)



(80a)



(80b)

The n-m-r. spectrum of the acetyl derivative of the methyl ester (Fig. 25) indicated the carbomethoxy group (methyl

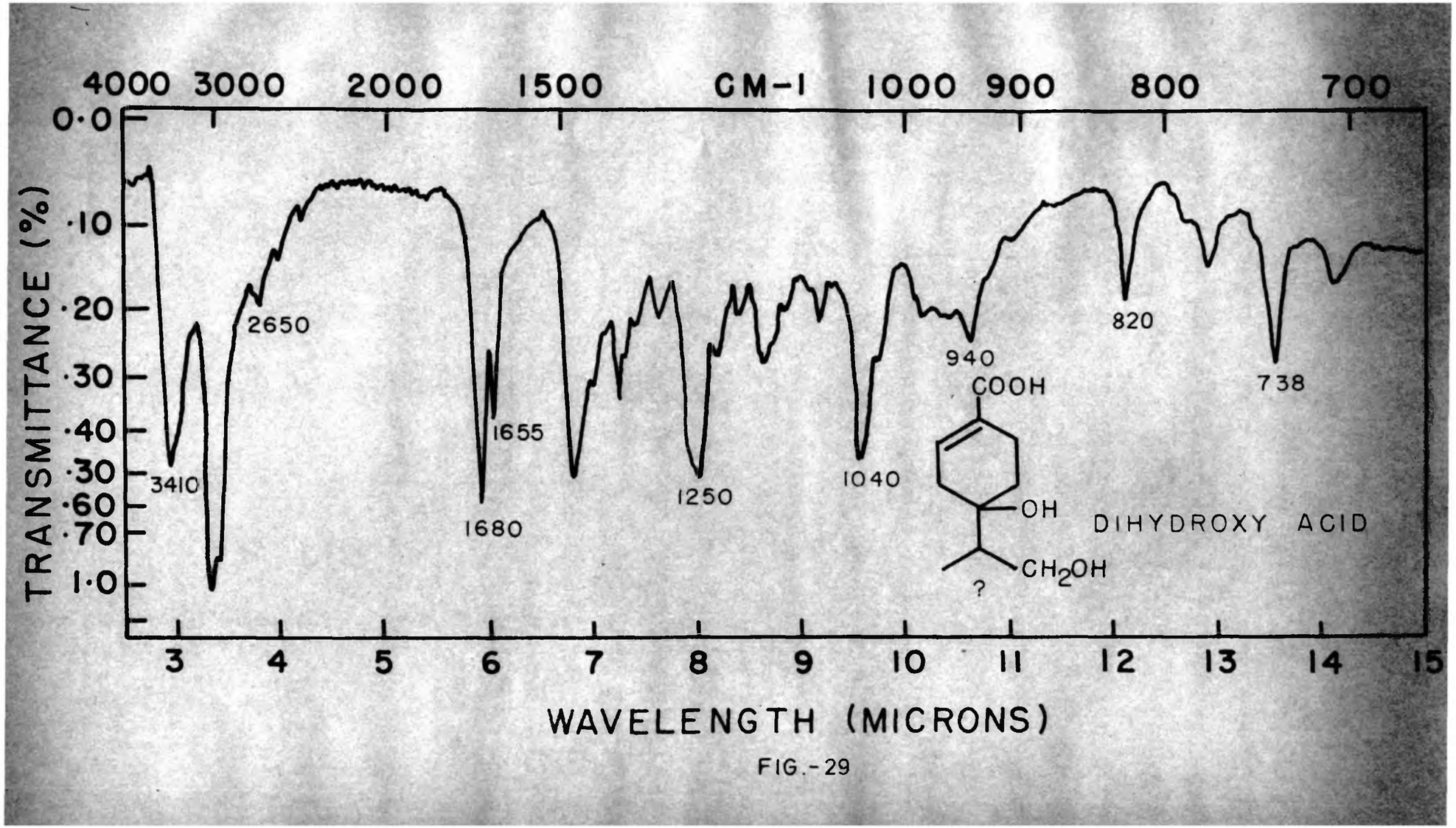

4000 3000 2000 1500 CM-1 1000 900 800 700
0·0
·10
·20
·30
·40
·30
·60
·70
1·0
TRANSMITTANCE (%)
3410
2650
1680
1655
1250
1040
940
820
738
COOH
OH
CH2OH
?
DIHYDROXY ACID
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG.-29

proton signal at 6.35 τ), two protons of an acetylated primary hydroxyl (6.05 τ), one vinyl proton in β-position to an α,β-unsaturated carbonyl system (3.13 τ), and a methyl group sitting on a carbon atom (split at 8.75-8.90 τ) carrying a hydrogen atom.

With structure 80b only one peak at 8.85 τ would have appeared for the methyl protons. Therefore structure 80 with the tertiary hydroxyl group at position 4 fits in with these data. It should be noted that dihydroxy acid (79) is metabolised by the limonene-grown cells (vide chapter on Growth and adaptive enzyme studies) whereas all other compounds tested such as oleuropeic acid, α-terpineol with a hydroxy group at position 8 are not metabolised by limonene grown cells. Furthermore, the compound did not react with periodate. The n-m-r spectra of the acid (taken in pyridine, not shown) and of the acetylated ester of the dihydroxy acid essentially support the structure 79 for the acid. However, further chemical work could not be undertaken due to paucity of material.

Auto-oxidation of limonene

In order to determine whether any of the above oxygenated compounds could be derived by auto-oxidation, experiments were run under identical fermentation conditions. After three days of incubation of limonene with the basal medium the products were extracted and separated into neutral and acidic components in the usual manner.

It was found that only trace amounts of acidic components were obtained in the acid fraction while in the neutral extract small amounts of oxygenated products could be detected. The neutral fraction was chromatographed over alumina and the fractions eluted with the usual solvent systems showed the presence of carvone (57), carveol (58), Diol 2 (62) and a small quantity of Diol 3 (64). Dihydrocarvone (56) and hydroxy ketone (60) could not be isolated in appreciable amounts. However the quantities of the neutral product isolated from the controls were almost insignificant when compared with those from the bacterial fermentation. Table 14 gives the amount of neutral and acidic products formed during auto-oxidation of limonene.

Experimental

[A] Isolation and characterization of a limonene-degrading bacterium

The microorganism used in the present investigation was isolated from the garden soil by enrichment culture techniques. Limonene and an ammonium salt were used as the sole source of carbon and nitrogen respectively. After seven to eight successive sub-cultures in the basal medium the organism was purified further by dilution and streak methods.

The organism was grown in a chemically defined medium (see Chapter II : Materials and Methods).

The organism isolated under these conditions was identified as belonging to the genus Pseudomonas according to Bergeys (Breed, 1957) Determinative Bacteriology. The microscopic characteristics and biochemical performances of the organism are presented in Tables 5 and 6.

[B] Propogation of culture

The cultures were propogated in nutrient agar slants (vide Chapter II - Materials and Methods).

Table - 5

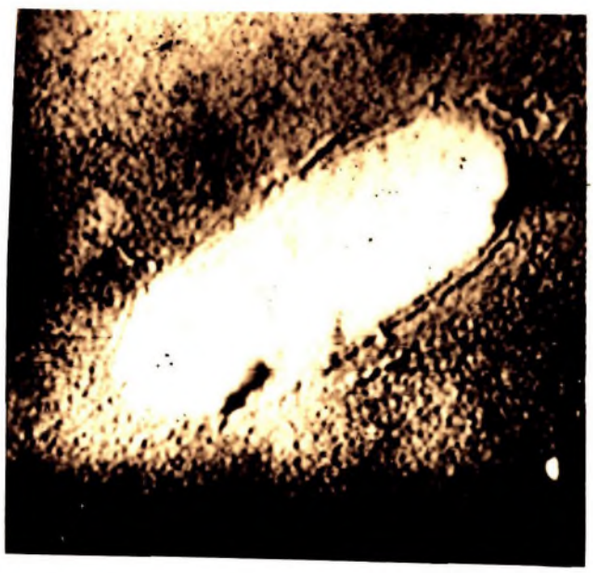
Sr. No.	Test	Observation
1.	Growth	aerobic, rod shaped
2.	Gram staining	remained negative at all stages.
3.	Sporulation	non-spore forming
4.	Dimensions	0.5 - 1.0 to 2 μ
5.	Flagella (see electron micrograph)	single flagellu monotrichous.
6.	Motility	motile
7.	Growth : 25°	++
	30°	++++
	37°	+
	45°	-
	55°	-
8.	Agar colonies 24 hr at 30°	Punctiform, flat, translucent smooth.
9.	Agar stroke 18 hr at 30°	Moderate, filiform, butyrous and no chromogenesis.
10.	Nutrient broth 18 hr at 30°	No surface growth, subsurface turbid, abundent, viscous.
11.	Nutrient Agar Slant 30° for 24 hr.	No fluorescence.
12.	pH-optimum	6.0 - 7.0
13.	Treatment at 56° for 10 min.	viable.

Table - 6

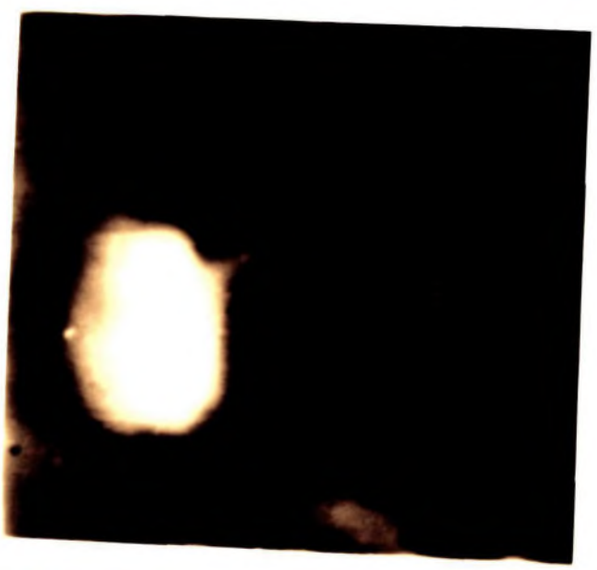
Biochemical Tests

Sr. No.	Test	observation
1.	Catalase	++++
2.	Methylene blue reduction	weak
3.	Nitrate reduction	+
4.	Hydrolysis of starch	+
5.	Indole	-
6.	Phenyl alanine	-
7.	Methyl red	-
8.	Acetyl methyl carbinol	-
9.	Indole-acetate test	yellow with and without
10.	Resistance to Penicillin	not sensitive
11.	NaCl tolerance	upto 6.5%
12.	NH_3 from peptone	+
13.	Growth at initial pH 5.5	+
14.	Citrate utilization	+
15.	Gelatin liquefaction	weak
16.	Asparagine utilization	+
17.	Urea utilization	-
18.	Ammonium sulphate utilization	-
19.	Milk	Peptonized, acidic, coloured.
20.	Acid and gas : Glucose, Xylose, Lactose, Maltose, Glycerol, Sucrose, Inositol, D-arabinose	All positive

Electron micrographs of limonene degrading bacterium



11000 x



9000 x

Preservation of the organism

The organism was incubated on nutrient agar slant at 28° for 24-30 hr until there was vigorous growth. Paraffin oil sterilized at 160° for two hours was added asceptically directly over the slants to a depth of about 2 cm above the tip of the slant. The culture was stored in duplicates in an upright position at 0°, 15° and 25° in a glass cabinet. No special precaution was taken excepting that the cotton plug was protected by polythene paper to lessen the danger of penetration by dust particles. One set of cultures was kept undisturbed, while the other was used for periodic transfers. Tests for viability were made by removing the material from the slant containing the paraffin oil with a sterile platinum loop. The oil was drained by drawing the inoculum along the inside of the same tube and the inoculum was plated on to a fresh agar slant. It was found that the organism was viable for at least six months.

[C] Nutritional requirements

The different substrates were examined singly in concentrations of 0.2 to 1% for their ability to replace limonene in the basal medium. Of these glucose, β-pinene, p-cymene, malate, succinate and citrate supported the growth much faster and approximately to the same extent as limonene. The growth on limonene in 24 and 48 hr respectively with various other substrates is presented in Table 7. α-pinene, terpineol, myrtenol, camphene, Δ^3 carene, cyclohexene,

longifolene, do not appear to support growth on limonene grown cells while glutamic acid, serine , valine, glycerol, maltose, arabinose, myrcene, ocemene support growth to some extent. With glucose grown cells, the growth on various substrates (except α-pinene) is practically the same.(Table 8).

[D] Effect of pH on the growth of the organism

The pH was found to be an important factor for the growth of the bacterium. The growth of the bacterium at pH 5 and 6 was considerably less than that at pH 7.0 or pH 8.0 with an optimum around pH 7.0 (Table 9). Moreover, the organism grew as an uniform suspension at pH 7.0 only. At a more alkaline or acidic pH there was widespread clumping of the cells.

[E] Effect of concentration of phosphate buffer on the growth of the organism

The effect of different ionic concentrations of phosphate buffer was studied on the growth of the organism. It was observed that (~~Table 4~~) a potassium phosphate concentration of between 0.012M and 0.036M in the basal medium was optimal for maximum growth whereas higher concentrations of phosphate in the range of 0.108 to 0.90M were inhibitory to some extent. (Table 10).

[F] Effect of concentration of substrate on the growth of the organism

In order to establish the optimal concentration

of limonene conducive to maximum growth of the organism graded levels of limonene were studied; With an increase in the concentration of the substrate there was an increase in growth. (Table 11). A plateau on the growth curve was reached with 0.6 ml of limonene per 100 ml medium. Even at 2.0% level limonene did not appear to inhibit the growth of the bacterium.

[G] Effect of concentration of salts on the growth of the organism.

The effect of salt concentration on the growth of the organism was studied. It was not considered necessary to define the optimal concentration of each of the constituents in the salt mixture and only the combined effect of all the ingradients was studied. Table (12) indicates that on decreasing the salt concentration (i.e. half of the normal) or increasing the salt concentration (viz. one and half times more than the normal) there was not much of a change in the growth of the organism. With lower concentrations the growth was less. (Table 12).

[H] Effect of concentration of organic acids on the growth of the bacterium.

The effect of concentration of organic acids on the growth of the organism was studied. It was found that with higher concentrations of substrate there was an

increase in the growth of the organism and a plateau on the growth curve was reached with about 0.8 ml of substrate per 100 ml medium. The growth of the bacterium was more in succinate than that ~~of~~ in melate or citrate. It was also observed that there was a wide spread clumping of the organism.

Limonene or glucose grown cells (24 hr old) from one flask were used for the experiment. The cells were centrifuged, washed with phosphate buffer (M/20) or glass distilled water and suspended in either phosphate buffer or glass distilled water and turbidity adjusted to 200 on Klett-Summerson photoelectric-colorimeter at 660 mμ. One ml of this suspension was used in the growth studies.

[I] Preparative-scale fermentation and extraction.

Limonene used in the present investigation was obtained from M/S B.D.H. Co. and S.H. Kelkar & Co. Bombay. It was purified by distillation by a batch-stripping distillation in a packed column. The fraction distilling at 170-178° was collected and redistilled. A colourless fraction distilling at 174-176° was collected [B.P.174-176°/ 712 mm, α_D^{27} + 104]. The material gave a single peak in the V.P.C. retention time 4.3 min at 100°, 4L/hr; succinic acid polyester.

Table (7). Growth of Bacterium on various substrates

Klett reading (660 mμ)

	Quantity g	control	Hours of Incubation 24 hr	48 hr
Limonene	0.20	40	328	315
α-Pinene	"	"	53	63
β-Pinene	"	"	307	322
Camphene	"	"	52	45
p-cymene	"	"	295	185
Myrcene	"	"	132	128
Δ^3 carene	"	"	70	55
Ocemene	"	"	105	110
Serine	"	"	228	215
Glutamic acid	"	"	70	165
Valine	"	"	192	215
Arabinose	"	"	88	250
Glucose	"	"	290	280
Sucrose	"	"	36* (280)**	34* (300)**
Maltose	"	"	226	220
Glycerol	"	"	205	225
Ammonium acetate	"	"	175	190

Inoculum : limonene grown cells (24 hr old) cell suspension in phosphate buffer M/20, turbidity reading 200 at 660 mμ ; 1 ml cell suspension and 0.2g of substrate per 100 ml medium used.

* It has been observed that after a lag phase the bacterium grows profusely on this substrate.

** 60 hr and 84 hr respectively.

Table (8). Growth of bacterium on various substrates

Klett readings (660 mμ)

Sr. No.	Substrate con. 0.2 g	Control	Hours of incubation	
			24 hr	48 hr
1	Limonene	44	350	345
2	α-pinene	"	230	218
3	β-pinene	"	276	262
4	β-alanine	"	218	180
5	Serine	"	200	182
6	Valine	"	214	216
7	Arabinose	"	185	246
8	Glycerol	"	254	246
9	Maltose	"	248	248
10	p-cymene	"	350	345
11	myrcene	"	128	125
12	Ocemene	"	165	182
13	Glucose	"	294	300

Inoculum : glucose-grown cells (20 hr old), cell suspension in phosphate M/20 buffer; turbidity reading 200 at 660 mμ;. 1 ml cell suspension + 0.2 g substrate added to 100 ml medium.

Table (9). Effect of pH on the growth of the bacterium

Klett reading (660 mμ)

pH of the medium	Control	Hours of incubation			
		12 hr	18 hr	24 hr	48 hr
5	40	75	63	65	55
5	"	62	64	64	57
6	"	70	108	125	130
6	"	72	135	145	147
7	"	108	187	187	180
7	"	112	190	190	182
8	"	86	137	120	117
8	"	93	157	152	147

Inoculum : limonene-grown cells (24 hr), cell suspension in phosphate buffer pH 7,(Turbidity reading 200 at 660 mμ), 1 ml cell suspension; 0.1 ml limonene/100 ml basal medium was used in the above growth studies.

Table (10). Effect of concentration of phosphate (K) buffer on the growth of the organism.

Klett readings (660 mμ)

Molarity of the potassium phosphate buffer M	Initial pH	Control	Hours of incubation					
			6 hr		12 hr		24 hr	
0.012	7	45	95	98	180	182	224	220
0.036	"	"	99	107	180	200	224	195
0.108	"	"	110	112	132	160	170	189
0.30	"	"	27	30	29	30	39	40
0.90	"	"	34	36	30	34	45	40

Inoculum : Limonene grown cells (24 hr old). cell suspension in water; turbidity reading 200 at 660 mμ ; 1 ml cell suspension + 0.1 ml limonene added to 100 ml basal medium

Table (11). Growth of organism with graded levels of limonene

Hours of incubation	Control	Limonene (ml)/100 ml medium													
		0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.5	0.7	0.9	1.2	1.5	2.0
24 hr	35	127	197	260	306	340	374	400	425	465	465	465	465	465	460
48 hr	30	120	186	242	290	320	355	377	398	435	467	510	565	600	598
72 hr	27	113	180	236	278	310	342	360	380	410	440	480	545	570	600

Inoculum : limonene-grown cells (24 hr old). Cell suspension in phosphate M/20 buffer. Turbidity reading 200 at 660 mμ . 1 ml cell suspension + different concentration of limonene added to 100 ml medium.

Table (12). Effect of salt concentration on the growth of the organism.

Klett reading (660 mμ)

Concentration of salts in 100 ml medium.	pH of the medium	Control	Hours of incubation			
			6 hr	24 hr	30 hr	48 hr
Half	7.0	40	36	305	330	305
*normal	7.0	45	55	325	360	350
one and half times	7.0	48	75	355	385	370

Inoculum : Limonene grown cells (24 hr old); cell suspension in phosphate buffer (M/20); turbidity reading 200 at 660 mμ , 1 ml cell suspension + 0.3 ml limonene added to 100 ml medium.

* normal salt concentration is given in media preparation - chapter II - Materials and Methods.

Table (13). Effect of concentration of organic acids on the growth of the bacterium

Klett reading (660 mμ)

Concn. %	pH of the medium initial	Control	Succi-nate	Malate	Citrate	pH of medium after 24 hr
0.1	7	45	154	125	105	7.3-7.4
0.2	"	"	250	192	165	7.8-8.0
0.4	"	"	345	300	284	9.3-9.4
0.6	"	"	425	370	340	9.5-9.6
0.8	"	"	430	415	375	9.4-9.5

Inoculum : Limonene-grown cells (24 hr old); cell suspension; turbidity reading 200 at 660 mμ ; 1 ml cell suspension and 0.2 ml limonene added per 100 ml medium. Incubation period 24 hr.

Fermentation and separation of products

In a typical fermentation 40 flasks, each containing 100 ml basal medium, were sterilized at 15 psi pressure 20 min. Four flasks containing limonene grown cells for 24 hr were centrifuged off in a refrigerated centrifuge at 0-2°. The cells were washed with M/20 phosphate buffer and suspended in 40 ml medium. 1 ml of this cell suspension and 0.3 ml limonene were added to each flask and the flasks incubated on a rotary shaker for 3 days (28-30°). Further additions of limonene, 0.4 ml and 0.3 ml, were done after 24 hours and 48 hours respectively (Total 1 ml = 0.84 g limonene added to each flask). After 72 hr of incubation, the contents of the flasks were pooled together, acidified with 6N HCl (20 ml) (pH 2.5 to 3), and extracted (1/10 the volume of the contents), and separated in the manner described in chapter II - Materials and Methods.

For isolation of the metabolites three batches of fermented medium (total 135 flasks) were pooled. The total ether extract (15 g) was separated into acidic (A_1) and neutral (N_1) components. The neutral components (N_1) were separated by Craig distribution into non-polar (N_1-I; 6.06 g) and polar (N_1-II; 2.17 g) fractions.

The butanol extract (.85 g) was also thus separated in neutral (N_2 ; ca 100 mg) and acidic (A_2 ; 0.8 g).

The broth after ether and butanol extraction was rendered alkaline with ammonium hydroxide (4N; pH 8.5-9.0)

as described in Chapter II and concentrated to a small volume (300 ml) and extracted with ether (500 ml) in a continuous extractor. The ether extract yielded the polar neutral fraction (N_3 ; 110 mg) on concentration. The extracted broth was then acidified with hydrochloric acid (6N) and extracted with ether as before. The acidic fraction (A_2) obtained after concentration gave a solid acid (240 mgs).

(1) Chromatography of the neutral fractions (N_1)

The neutral fraction (N_1-I; 6.06 g) was chromatographed over grade I alumina (120 gm) and eluted with the usual solvent system.

The first fraction (N_1-I-i) eluted with pet ether was a liquid. It was distilled under reduced pressure. The fraction distilling at 90-95°, 1 mm was unreacted limonene (5.0 g) (55). The residual liquid (0.5 g) distilling at 180-190° bath 1 mm Hg was a fatty material.

The 2nd fraction (N_1-I-ii) eluted with 10% ether showed 2 spots in TLC (10% ethyl acetate in hexane, R_F 0.80 and 0.78 approx.) (350 mg). I.R. spectrum showed bands at 1712, 1675, 1645, 840 cm^{-1}, α,β-unsaturated and saturated ketone. UV λ max 236 mμ , ε 10,000; VPC two peaks in the ratio of 55:45 with retention time 8 min and 10 min respectively at 140° poly succinic ester.

The separation of these two components was achieved as follows : The mixture (350 mg) was taken in 5 ml of 10% sodium sulphite soln. and kept on a water bath (50-60°) with vigorous stirring . 1-2 drops of phenylphthalein was added and acetic acid (1N) was added drop by drop till the colour changed to red. The reaction mixture was cooled to room temperature and extracted with ether (15 ml x 3). After working up a liquid (150 mg) was obtained which was purified by distillation in vacuo. B.P.85 ± 2 (bath) at 0.75 mm; η_D^{30} 1.4720. I.R. ν_{max} 1712, 1645, 1450, 1378, 1320, 1267, 1250, 1220, 1182, 1142, 1110, 1085, 1055, 1020, 958, 890, 840, 797 cm^{-1} (Fig.8). (Found : C = 79.03, H = 10.42, calc. for $C_{10}H_{16}O$: C, 78.89; H, 10.59%); VPC retention time 7.5 to 8 minutes; 140°, polyester succinic acid 700:100, 1.7/hr.

The sodium sulphite adduct was rendered alkaline in the presence of ether (10 ml) with sodium carbonate (5%) and extracted with ether (10 ml x 3). After working up the α,β-unsaturated ketone (57) was obtained (150 mg); b.p. 85-90° (bath) at 0.75 mm; η_D^{32}, 1.4920; $[\alpha_D^{27}]$ + 59; UV absorption at λ max 235-236 mμ ; ϵ -18340. I.R. ν_{max} 1675, 1645, 1450, 1428, 1365, 1245, 1140, 1112, 1057, 1050, 1027, 1000, 960, 890, 800, 703 cm^{-1} (Fig.9); TLC (10% ethyl acetate in hexane, R_F 0.75; VPC, retention time 10 minutes on 140° polyester succinic acid 700:100, 1.7/hr. (Found : C, 80.34, H, 9.69; $C_{10}H_{14}O$ requires : C, 79.95, H, 9.35%).

2:4-dinitrophenylhydrazone of ketone (57)

Ketone (57)(30 mg) was dissolved in 1 ml methanol containing 1 drop of sulphuric acid and was refluxed with 1% alcoholic, 2:4-dinitrophenylhydrazine for 5 minutes. The clear orange solution kept for two hours at 6-8°. The compound that separated out was filtered on a Buchner, washed with dil. methanol, and dried in vacuo (50 mg). After recrystallization from methanol the compound melted at 183-184°. (literature m.p. of 2:4-dinitrophenyl hydrazone of carvone 187°) (Pines, 1955)

Monohydroxy alcohol (58)

The next fraction from the column (N_1-I-iii) which was eluted with 100% ether was a liquid (100 mg). B.p. 90-95 (bath) 0.75 mm. η_D^{30} 1.4940, $[\alpha]_D$ + 21. I.R. ν_{max} 3355, 1642, 1440, 1372, 1080, 1057, 1030, 888, 810 cm^{-1} (Fig. 10). (Found : C, 78.18, H, 10.65, calc. for $C_{10}H_{16}O$: C, 78.89; H, 10.59%).

Oxidation of alcohol (58) with manganese dioxide

To the alcohol (58) (32 mg) 2 ml chloroform was added and the contents refluxed for 1 hr with 35 mg of active manganese dioxide. (Manganese dioxide was prepared according to the method of Attenburrow et al, 1952). After the reaction was over, the manganese dioxide was filtered using hyflosupercel. The filtrate after concentration gave a

liquid (25 mg) I.R. γmax 1670, 1642, 892 cm^{-1}. TLC 5% ethyl acetate in hexane. R_F 0.72 compared with that of authentic sample of carvone.

The last fraction (N_1-I-iv) (30 mg) eluting out with methanol contained polar fractions. This fraction was pooled with the more polar neutral fraction - 90% methanol (N_1-II).

(2) Chromatography of neutral fraction - 90% methanol (N_1-II)

The polar neutral fraction (N_1-II, 2.2 g) was chromatographed over alumina (40 gm) and eluted with the usual solvents.

The 1st fraction (N_1-II-i) eluted with pet ether was a liquid (15 mg) and showed 3 spots in TLC (R_F 0.80, 0.76, 0.6, 10% ethyl acetate in hexane) which were identified as dihydrocarvone (56), carvone (57) and carveol (58) by a comparison with the authentic samples.

The 2nd fraction (N_1-II-ii) eluted with 5% ether was a liquid (59) (90 mg). B.p. 100-105° (bath) at 1 mm. The sample consumed one mole of periodate in 5 min. at 28°. I.R. γmax 3355, 1660, 1449, 1374, 1120, 1052, 1032, 888, 810 cm^{-1} (not shown), $[\alpha]_D$ = + 26.7. (Found : C, 70.0, H, 10.5%; $C_{10}H_{18}O_2$ requires : C, 70.54; H, 10.66%).

Acetylation of Diol (1) (59)

To diol 1 (59) (17 mg) pyridine (0.2 ml) and acetic anhydride (0.25 ml) was added and the contents kept at 25° temp. for 16 hr. After working up, a monoacetylated compound was obtained. B.p. 90-95° bath, 1 mm; $[\alpha]_D$ = + 35. I.R. νmax 3540, 1750, 1650, 1450, 1375, 1240, 1047, 887, 835, 804 cm^{-1}. (Found : $COCH_3$, 17.4%; one $CO-CH_3$ requires : 20,28%).

Isolation of hydroxy ketone (60)

The 3rd fraction (N_1-II-iii) eluted with 10%-50% ether was a slightly viscous liquid (250 mg). TLC showed 2 spots, 10% ethyl acetate in hexane, R_F 0.5 and 0.47; proportion 85:15.

To the fraction (N_1-II-iii) (250 mg) pet ether (2 ml) was added and the contents kept in a test tube in ice bath. Carbon tetrachloride (2 ml) was added. On scratching the sides of the tube, colourless crystals separated (35 mg; m.p. 64°). The mother liquor was concentrated and distilled to give the hydroxyketone (60) (190 mg). B.p. 110-120° bath, 2 mm. I.R. νmax 3450, 1700, 1645, 1450, 1375, 1175, 1125, 1050, 1032, 910, 887, 850, 800, 732 cm^{-1} (Fig.12). (Found : C, 71.03, H, 10.02%; $C_{10}H_{16}O_2$ requires : C, 71.39, H, 9.59%). VPC retention time 12 minutes at 140° succinic polyester. The compound consumed one mole of periodate. The periodate reaction mixture

(containing 30 mg of the hydroxy ketone and 1 m.mole periodate in 5 ml 50% alcohol) after 30 min was acidified and extracted with ether (10 ml x 3). The ether extract on evaporation gave a keto acid (25 mg) (61). I.R. νmax. 2630, 1708 cm^{-1} (not shown). The keto acid gave a positive iodoform test.

Isolation and characterization of Diol 2 (62)

The fourth fraction (N_1-II-iv) was one of the major fractions obtained from the column. It was eluted with 50-100% ether. It was a viscous colourless liquid (608 mg) and was always contaminated with the hydroxy ketone (60) and Diol 3 (64).

Purification of Diol 2 (62)

The fraction (N_1-II-iv) 808 mg was dissolved in pet ether (5 ml) and chilled in an ice bath. Carbon tetrachloride was (2.3 ml) added slowly and crystallization was induced by scratching.. Diol 2 (62) (600 mg) crystallized out. After recrystallization from carbon tetrachloride the diol 2 (62) (592 mg) melted at 64°. TLC-single spot; (15% ethyl acetate in hexane R_F 0.45, VPC retention time 19.5 min at 140°, 700:100 polyester succinic acid; b.p.155-160° bath, 0.5 mm. I.R. νmax 3475, 1637, 1450, 1370, 1290, 1255, 1236, 1180, 1158, 1128, 1050, 1037, 1000, 960, 945, 910, 888, 863, 820, 790, 725 cm^{-1} (Fig. 13). UV absorption λ max 209 mμ, ϵ 3600. $[\alpha]_D^{27}$ = + 29.1. (Found : C, 70.06, H, 10.6%;

calculated for $C_{10}H_{18}O_2$: C, 70.54, H, 10.66%). Lit. (Schmidt 1949) reports for p-menth-8-ene-1,2-trans-diol m.p. 65°.

Acetylation of Diol 2 (62)

Diol-2 (62) (45 mg) was dissolved in dry pyridine (0.5 ml) and treated with acetic anhydride (0.5 ml) in pyridine (0.4 ml). The mixture after working up gave a product distilling at b.p. 120° (bath) 1.5 mm (50 mg). I.R. νmax 3560, 1750, 1645, 1450, 890 cm^{-1} (not shown).

Chromium trioxide oxidation of Diol 2 (62)

To Diol 2 (7 mg) in dry pyridine (0.1 ml), chromium trioxide (10 mg) in pyridine (0.1 ml) was added with shaking. The reaction mixture was kept overnight at 25°. Next day after decomposition of excess chromium trioxide with 2-3 drops of methanol the mixture was diluted with 5 ml water and extracted with ether (5 ml x 3). The ether extract was washed with aq. bicarbonate (2 ml x 1) and finally with brine, and evaporated to yield 5 mg of hydroxy ketone. I.R. spectrum identical with that of the hydroxy ketone (60) (Fig.12).

Isolation and characterization of Diol-3 from (N_1-II-v)

When the column was eluted with 10% ether and finally with methanol a fraction (1 g) (N_1-II-v) consisting of a viscous liquid was eluted out ~~(1 g)~~. Pet ether (5 ml)

was added to this fraction and the solution was chilled. Carbon tetrachloride (5 ml) was added and a solid Diol 2 (62) removed as described previously. The mother liquor containing Diol 3 (64) was further purified by distillation in vacuo. B.p. 155-160°. (bath) 1 mm. $[\alpha]_D^{27}$ = + 85. $\eta_D^{34.5}$ 1.4950. I.R. νmax : 3475, 1637, 1450, 1370, 1290, 1255, 1236, 1180, 1158, 1128, 1050, 1037, 1000, 960, 945, 910, 888, 863, 820, 790, 725 cm^{-1} (Fig. 15). (Found : C, 70.01, H, 10.73%; $C_{10}H_{18}O_2$ requires : C, 70.54, H, 10.66%). Diol 3 did not take up any periodate.

Acetylation of Diol 3 (64)

To Diol 3 (64) (40 mg) pyridine (0.8 ml) and acetic anhydride (0.5 ml) were added, and the mixture was kept overnight at 25°. After working up in the usual manner the product obtained was a liquid (56 mg); b.p. 120-125° (bath) 4 mm. I.R. νmax 1725, 1650, 1440, 1370, 1250, 1170, 1120, 1030, 1000, 950, 920, 860, 802, 790, 740 cm^{-1} (not shown).

Oxidation of Diol 3 (64) with manganese dioxide

To Diol 3 (64) (35 mg) carbon tetrachloride (2 ml), active manganese dioxide (35 mg) were added and the mixture refluxed for 1 hr. It was filtered and the precipitate was washed with carbon tetrachloride and the filtrate after evaporation of the solvent distilled in vacuo. B.p. 110-115° (bath) - 4 mm. UV λ max 236 mμ. I.R. νmax 3350, 1685, 1645,

1445, 1410, 1325, 1240, 1172, 1080, 1030, 850, 801, 740, 730 cm^{-1}.

(3) Acidic Components

The acidic components (A_1) extracted from the first ether extract in aqueous sodium carbonate (5%), were liberated by acidification and extraction with chloroform. The chloroform extract after concentration gave an oily product (5.27g). Thin layer chromatography revealed at least 4 spots (Benzene, methanol, acetic acid 22:2:1; R_F 0.7, 0.5, 0.38, 0.3). The total fraction (5.27 g) was converted into methyl esters with diazomethane. The methyl esters (5.60 g) (A_1Me) were subjected to four transfer partitioning between light petroleum and 90% methanol. The methyl esters in pet ether fraction (A_1Me-I; 0.9 g) and methyl esters in 90% methanol fraction (A_1Me-II; 4.6 g) were separately chromatographed over alumina.

(3a) Chromatography of methyl esters (A_1Me-I)

The methyl esters (A_1Me-I; 0.9 g) were chromatographed over alumina (25 g) and eluted with the usual sequence of solvents.

(i) Fatty acid ester (pet ether fraction)
(ii) Monocarboxylic ester (2-10% ether)
(iii) Perillic acid methyl ester (10-25% ether)
(iv) Traces of dicarboxylic acid (50-100% ether).

The first fraction (A_1Me-I-i) eluted with pet ether was a liquid (0.50 g). B.p. 180-190° (bath) 3 mm. I.R. and analysis (not given) indicated that the compound was a fatty acid ester.

The 2nd fraction (A_1Me-I-ii) eluting out of the column with 2-10% ether was also a liquid (70 mg). TLC - 10% ethyl acetate in hexane gave a single spot (R_F 0.72) but VPC indicated that the fraction was a mixture of two components (40:60) with retention time 6 and 8 min. at 160°.

Saponification of the ester (A_1-Me-I-ii)

The ester (70 mg) was saponified with 5 ml, 4N ethanolic potassium hydroxide for overnight. The mixture was diluted, acidified and extracted with ether (10 ml x 3). The free acid after working up was distilled. B.p. 105-110° (bath) 1 mm (56 mg.). Thin acid was converted into its cyclohexylamine salt, recrystallized from acetone; m.p. 110°. I.R. ν_{max} 2650, 2220, 1650, 1530, 1440, 1370, 1250, 1130, 1000, 900 cm^{-1} (spectrum not shown). The salt (66 mg) was decomposed with 6N HCl and acid extracted with ether (10 ml x 3). The ether extract after concentration gave a liquid acid (38 mg), b.p. 105-110°. (bath) 1 mm. I.R. ν_{max} 2650, 1700, 1618, 1430, 1413, 1370, 1280, 1240, 1220, 1190, 1150, 1055, 1012, 960, 935, 910, 845, 800, 760, 692 cm^{-1}. (Found : C, 71.88, H, 9.79%; $C_{10}H_{16}O_2$ requires : C, 71.42, H, 9.55%).

Isolation of perillic acid methyl ester (67)

The third fraction eluting from the column (A_1Me-I-iii) with 10-25% ether was a colourless liquid (350 mg). b.p. 110-115° (bath) 2 mm. U.V. λ max 218 mμ; ϵ 8107; $n_D^{31.5}$ 1.4835; $[\alpha]_D^{27}$ + 101.7. I.R. νmax 1710, 1645, 1430, 1370, 1312, 1280, 1250, 1200, 1140, 1180, 1040, 970, 940, 920, 890, 858, 803, 788, 743, 707 cm^{-1} (Fig. 17). Bands at 1710, 1615 cm^{-1} (α,β-unsaturated methyl ester). TLC single spot 10% ethyl acetate in hexane; R_F 0.7, VPC-single peak, retention time 4 minutes at 195° Poly Az column. 160 m. amp. 8 sec/10 ml. (Found : C, 71.15; H, 9.39%; calculated for $C_{11}H_{16}O_2$: C, 70.30; H, 8.95%).

Saponification of the methyl ester (67)

Compound 67 (200 mg) was saponified with methanolic KOH (1 ml) at 25° for overnight. The unreacted methyl ester was extracted with ether (10 ml x 3) from diluted reaction mixture and the alkaline extract after acidification with 6N HCl was extracted with chloroform (10 ml x 3). On evaporation of the chloroform solution the compound separated as colourless plates. The acid (66) was crystallized from pet ether m.p. 123-124°, and purified by sublimation in vacuo (2 mm, 82°C), (150 mg); m.p. 124-125°; UV λ max. 216 mμ ; ϵ 8466; $[\alpha]_D$ + 89. I.R. νmax : 2650, 1680, 1645, 1450, 1430, 1370, 1312, 1275, 1200, 1142, 1080, 1036, 940, 890, 778, 737, 700 cm^{-1} (Fig. 16). (Found : C, 72.3, H, 8.5% calc. for $C_{10}H_{14}O_2$ C, 72.26, H, 8.49%). Neutral equivalent 166 ± 2 $C_{10}H_{14}O_2$

requires 166. I.R. spectrum identical with an authentic synthetic perillic acid (Fig. 15). There was no depression in the mixed m.p.

The 4th and the last fraction (A_1Me-I-iv) eluting from the column with 50-100% ether was obtained only in a very small quantity ~ 30 mg. I.R. spectrum and analysis indicated that it was a dicarboxylic acid ester. It was pooled with the methanol fraction from the Craig (A_1Me-II).

(3b) Chromatography of the methyl esters in 90% methanol fraction (A_1Me-II)

The mixture of methyl esters (4.6 g) obtained from methanol layer (A_1Me-II) was chromatographed on 120 gm of alumina and eluted with the usual sequence of solvents.

The 1st fraction (A_1Me-II-i) eluting out in pet ether was a liquid (488 mg). Its I.R. spectrum corresponded with that of a methyl ester of a C_{18} fatty acid.

Isolation of perillic acid methyl ester (67)

The 2nd fraction (A_1Me-II-ii) eluting with 5% ether was mainly a monocarbomethoxy compound (67) (664 + 50 mg) b.p. 108-110° (bath) 2 mm (620 mg). It was identified as perillic acid methyl ester through VPC, and comparative I.R. spectroscopy

Isolation of the dicarboxylic acid methyl ester (69)

Pet-ether-ether mixtures (10-50%)eluted a fraction (A_1Me-II-iii) in substantial amounts (1.515 g) b.p. 125-130° at 0.75 mm. $n_D^{30.5}$ 1.4500, $[\alpha]_D^{28} \pm 0$. VPC retention time 7 min at 195°; 100:2 polyaze, 160 m.amp. 8 sec./10 ml. TLC 10% ethyl acetate, in hexane; R_F 0.5. I.R. ν_{max} 1740, 1643, 1440, 1380, 1360, 1250, 1200, 1170, 1082, 1016, 993, 900, 870, 845, 750 cm^{-1} (Fig.19). (Found : C, 62.85, H, 8.98%; $C_{12}H_{20}O_4$ requires : C, 63.13, H, 8.83%). It did not show any characteristic UV absorption in the range 210-400 mu .

Saponification of the diester (69)

The diester (69) (580 mg) was saponified with methanolic KOH (5 ml) at 25° for 24 hr and then on the steam bath for 30 min. The mixture was then cooled, diluted with 50 ml water and extracted with ether (15 ml x 2) to remove the unreacted diester. (a few mg). The aqueous alkaline layer was acidified with 6N HCl and then extracted with chloroform (25 ml x 3). After working up the solvent layer a thick viscous yellow acid (68) was obtained (500 mg) which was purified by repeated distillation. B.p. 195-200°(bath), 0.2 mm. η_D^{32} , 1.4635, $[\alpha]_D^{27} \pm 0$. I.R. ν_{max} 2650, 1710, 1645, 1460, 1410, 1365, 1285, 1240, 1200, 1160, 1080, 1055, 1020, 940, 898, 721 cm^{-1}. (Fig. 21). (Found : C, 60.5, H, 8.0%; $C_{10}H_{16}O_4$ requires : C, 59.98, H, 8.05%). Neutralization equiv. 101 ± 2, dibasic acid. $C_{10}H_{16}O_4$ requires : 100, TLC -

Benzene : Methanol : acetic acid (22:2:1); R_F 0.52*. *The spot appeared only when the plate was heated to 140-150°.

Cyclohexylamine salt of dicarboxylic acid (68)

The dicarboxylic acid (68) (250 mg) was dissolved in dry acetone (2 ml) and added to freshly distilled cyclohexylamine (1 ml) in acetone (1 ml). The contents were then kept at 3 to 4° for 6-8 hours. The crystalline precipitate was filtered in the cold and washed with cold acetone. The colourless crystalline material was recrystallized from acetone (2 ml). m.p. 148-150°. (200 mg - 1st crop) (20 mg - 2nd crop). I.R. ν_{max} 2680, 2580, 2220, 1645, 1490, 1450, 1380, 1300, 1240, 1195, 1075, 1048, 1020, 973, 923, 890, 845, 717 cm^{-1} (Fig. 22).

Attempted anhydride formation with compound 68

The dicarboxylic acid (68) (125 mg) was dissolved in acetic anhydride (0.5 ml) and refluxed for 1 hr and then slowly evaporated under reduced pressure. The acetic anhydride was trapped in a chilled trap and the residue was distilled. The product distilling at 190-195° (bath) 0.5 mm (80 mg) was collected. I.R. ν_{max} : 1815 (small shoulder), 1770, 1730, 1648, 1570, 1440, 1370, 1235, 1175, 1060, 1000, 950, 896 cm^{-1} (not shown). I.R. spectrum indicated the formation of a mixed anhydride.

Dieckmann's cyclization of Diester 69

The diester (69) (500 mg) was added slowly into a freshly prepared sodium ethoxide (13 mg) and refluxed for 6 hr. The contents were acidified with 33% acetic acid (3 ml) and extracted with ether. (5-7 ml x 5). The total extracts were concentrated and chromatographed on TLC (15% ethyl acetate in hexane; 2 spots; R_F 0.65 and 0.53). VPC retention time 0.5 and 2.2 min at 200°; 150 m. amps 9 Secs/10 ml. Polyester; relative proportion 70:30. The Dieckmann cyclization products (418 mg) were chromatographed over alumina (16 g). The unreacted dicarboxylic ester was first eluted out with 20-25% ether. The mixture (70, 71) of keto esters (160 mg) were eluted with 25-50% ether, b.p. 100-105°, (bath), 1 mm. I.R. νmax 1750, 1725, 1660, 1620, 1450, 1400, 1370, 1300, 1275, 1220, 1150, 1125, 1100, 1062, 1000, 892, 830. (not shown).

Reduction of keto esters (70,71) to hydroxyesters (73,74) by sodium borohydride

The mixture of keto esters (70 and71) were subjected to reduction with sodium borohydride. The keto esters (200 mg) were dissolved in methanol (1 ml) treated with 60 mg sodium borohydride and kept overnight at 25°. Next day the reaction mixture was carefully acidified with aqueous acid and the mixture was extracted with ether. (15 ml x 3). The mixture of hydroxy acid esters (73 and 74) (190 mg) obtained,

was distilled in vacuo. b.p. 150-175°. 1 mm. VPC-2 peaks, retention time 7.5 and10 min at 200°, 150 m.amps. 9.5 sec/ 10 ml. polyesters. One of the hydroxy ester (73) had identical retention time with the hydroxy acid ester (73) obtained by the fermentation of limonene. I.R. νmax 3500, 1728, 1725, 1450, 1370, 1300, 1260, 1175, 1100, 1060, 1050, 990, 965, 890, 792, 760, 710 cm^{-1} (not shown).

Hydrolysis and decarboxylation of β-keto esters 70 and 71

The mixture of keto esters (70 and 71) (120 mg) was refluxed with 10% sulphuric acid (10 ml) for 4-6 hours. The mixture was then cooled, diluted with water and extracted with ether (5 ml x 4). The total ether extract was washed with 5% sodium bicarbonate, concentrated and distilled to yield the ketone (85). b.p. 105-110° - 2 mm. I.R. νmax 1680 sharp peak, 1645, 1470, 1375, 893 cm^{-1} (not shown).

Hydrogenation of the dimethyl ester (69)

Dimethyl ester (69) (152 mg) was hydrogenated in 20 ml dry ethanol in the presence of Pt. catalyst (60 mg) for 4 hr. After the hydrogenation was over, the mixture was filtered on a buchner funnel using a hyflosupercel bed, and the bed was washed with ethanol. The filtrate was concentrated and distilled in vacuo to get a viscous liquid (75; 142 mg). b.p. 120-125° - 1 mm. η_D^{30} 1.44. I.R. νmax 1750, 1465, 1440, 1365, 1330, 1250, 1200, 1170, 1100, 1080, 1018, 990, 870, 835, 760, 704 cm^{-1}. (Found : C, 64.18, H, 9.70%;

$C_{12}H_{22}O_4$ requires : C, 62.58, H, 9.63%). This ester was identical in all respects with β-isopropyl pimelic acid dimethyl ester obtained by Bhattacharyya et al (1964) from fermentation of α and β pinenes by a Pseudomonad.

Ozonolysis of the diester (69)

Diester (69) (202 mg) was ozonolysed in ethyl acetate (20 ml) for two hours. After ozonolysis (starch iodide test) the solvent was removed in vacuo and trapped on a chilled trap containing water. The ozonolysis residue was dissolved in ether (10 ml) and was extracted with 5% bicarbonate (5 ml x 2) to remove any acid formed during ozonolysis. The keto ester (76) from the ether layer (180 mg , TLC - 30%, ethyl acetate R_F 0.3) containing some unreacted diester (69) (R_F 0.6) was chromatographed over alumina (4 gm). The fraction (76) eluting out with 25-50% ether was collected, (150 mg) and distilled b.p. 140-145° - 1 mm. I.R. ν_{max} 1732, 1710, 1660 cm^{-1}. Compound (76) gave a positive iodoform test indicating that it was a methyl ketone. The contents of the chilled receiver when treated with an alcoholic solution of dimedone (50 mg in 2 ml) yielded the formaldehyde derivative of dimedone (20 mg) which crystallized in its characteristic needle from m.p. 186-187°, mixed m.p. with an authentic sample 186°.

Chromatography of the hydroxy acid esters (A_1Me-II-iv)

Following the diester (69) from the column, the

fraction eluted with 75-100% ether (A_1Me-II-iv) was a liquid (960 mg) containing at least three components. TLC 20% ethyl acetate in hexane, three spots, R_F 0.5, 0.4, 0.32; relative proportion ~ 2:50:15. Separation of these esters was difficult by fractional distillation. The column was further eluted with 100% chloroform and 100% ethanol (290 + 153 mg). All these fractions were pooled and subjected to rechromatography.

Isolation of the hydroxy esters (73, 86)

The hydroxy acid methyl esters (1320 mg) were chromatographed over alumina (30 gm) and eluted with the usual solvent systems.

A small quantity of a liquid (50 mg) was eluted with 10% ether and showed a spot corresponding to the diester, R_F 0.5 on TLC. This was not further studied. The 25-50% ether eluates gave a liquid (750 mg) containing the hydroxy acid methyl ester (73) and traces of more polar esters and was purified by fractional distillation. b.p. 140-145°(bath), 1.5 mm, η_D^{30} 1.4840, $[\alpha]_D^{28}$ + 49; I.R. νmax 3600, 1750, 1650, 1440, 1375, 1258, 1212, 1170, 1040, 1020, 1000, 960, 892, 847, 804, 770, 718 cm^{-1} (Fig. 24) (Found : C, 65.72, H, 9.32%; $C_{11}H_{18}O_3$ requires : C, 66.64, H, 9.15%). [This hydroxy ester (73) had identical retention time in VPC with one of the hydroxy ester (73) obtained by the reduction of the Dieckmann cyclization products (70, 71) of the diester (69),

This compound (73) is also formed during perillic acid fermentation (vide Chapter on "growth and adaptive enzyme studies)].

Saponification of the hydroxy ester (73)

The hydroxy ester (73) (80 mg) was saponified with 0.5 ml of 4N methanolic potassium hydroxide overnight on a steam bath after dilution and acidification the free acid (77) (60 mg) recovered in ether and purified by distillation. b.p. 205-210° (bath) at 0.25 mm; neutral equivalent 180 ± 2, $C_{10}H_{16}O_3$ requires : 184; I.R. νmax : 3460, 2650, 1725, 1640, 1480, 1375, 1255, 1238, 1225, 1150, 1070, 1020, 945, 910, 890, 835, 800, 760 cm^{-1}. (Fig. 26).

Isolation of the hydroxy ester (86)

The next fraction coming out from the column with 50-100% ether was a yellow and slightly viscous liquid (300 mg) (TLC-R_F 0.3, ethyl acetate 15%), b.p. 160-165° (bath) 0.75 mm, η_D^{29} 1.4590. U.V. absorption λ max 218 mμ, ϵ 11700; indicating α,β-unsaturation. I.R. νmax 3410, 1708, 1650, 1440, 1380, 1310, 1260, 1220, 1200, 1170, 1090, 1042, 905, 808, 772, 750, 718 cm^{-1}. (Fig. 28). (Found : C, 66.5, H, 9.3%; $C_{11}H_{18}O_3$ requires : C, 66.64; H, 9.15%).

The last fraction (110 mg) eluting from the column with 100% chloroform and 100% ethanol was a viscous

yellow coloured liquid of higher polarity. TLC (30% ethyl acetate) displayed at least two spots, R_F 0.3 and 0.2. Due to unstable nature and paucity of the material further work on this fraction was not possible.

(3c) Acidic fractions from Butanol extract (A_2)

The acidic fraction obtained from the butanol extract (A_2; 837 mg) showed practically one spot corresponding to the dicarboxylic acid (68). (TLC, Benzene : Methanol : acetic acid, 22 : 2 : 1, R_F 0.52). It was therefore therefore converted into cyclohexyl amine salt (m.p. 148-150°). I.R. spectra of this product was superimposable with that of the cyclohexyl amine salt of the dicarboxylic acid (68) (Fig. 22).

Isolation and characterization of the polar acid (79)

This acid (79) was obtained from the broth by continuous ether extraction. The neutral fraction (N_3, 110 mg) was not worked up. The broth was then acidified with 6N HCl and extracted with ether continuously in an extractor for 72 hr. The solid that separated out (80 mg) was crystallized from ether. m.p. 180-182°. TLC single spot; Benzene : methanol : acetic acid, 22 : 2 : 1, R_F 0.11, U.V. absorption λ max 216 mμ, ϵ 12800 corresponding to an α,β-unsaturated acid. I.R. νmax 3410, 2650, 1680, 1655, 1470, 1440, 1400, 1318, 1250, 1160, 1040, 940, 825, 775, 738, 705 cm^{-1}. (Fig. 29). Neutral equivalent, 202 ± 2 :

$C_{10}H_{16}O_4$ requires : 200. (Found : C, 60.98, H, 8.34% ; $C_{10}H_{16}O_4$ requires : C, 59.98, H, 8.05%). This acid is insoluble in ether, chloroform, carbontetrachloride.

Methyl ester of the dihydroxy acid (79)

The acid (79) (40 mg) was converted into its methyl ester by diazomethane by the usual method. The methyl ester (80) (42 mg) was distilled. b.p.210-215° (bath) at 1 mm; the distillate solidified, m.p. 108-110°. I.R. νmax 3320, 1690, 1645, 1440, 1327, 1318, 1250, 1218, 1190, 1150, 1082, 1040, 990, 942, 928, 913, 970, 838, 810, 785, 742, 717, 710 cm^{-1}. (not shown).

Acetylation of the dihydroxy acid methyl ester (80)

The dihydroxy acid methyl ester (80) (30 mg) was dissolved in dry pyridine (0.3 ml) and added to acetic anhydride (0.5 ml) in pyridine (0.3 ml). The mixture was kept at 25° for 24 hr. The excess of pyridine and acetic anhydride were removed in vacuo, and the residual liquid containing the acetylated product, (35 mg), Compound (80b), was distilled in vacuo. b.p. 180-185° (bath) at 1 mm. the distillate solidified m.p. 42°. (Found : $COCH_3$, 15.0%; requires : 14.02% for one $COCH_3$).

Table (14). Neutral and acidic transformation products obtained by the auto-oxidation of limonene.

	No. of flasks	Incubation period hr	Neutral fraction g	Acidic fraction g
Auto-oxidation (Limonene alone)	20	72	0.170	10-12 mg
Experimental (Limonene + Bacterium)	20	72	0.50	1.04 g

CHAPTER - VI

GROWTH AND ADAPTIVE ENZYME STUDIES

GROWTH AND ADAPTIVE ENZYME STUDIES

DISCUSSION

From the variety of products obtained by the oxidation of limonene by the pseudomonad it would appear that there are more than one pathway for degradation of limonene by the organism. In order to ascertain the nature of these pathways, growth and adaptive enzyme studies were taken up with limonene; perillic acid and sugars grown cells.

It was observed that limonene grown cells could not grow on carvone (57), dihydrocarvone (56), Diol 2 (62) Diol 3 (64), hydroxy ketone (60), carveol (58); Neither do these compounds show any significant oxygen uptake with limonene-grown cells.

It is not always easy to interpret such data, as the permeabilities of these compounds into the cells are not known (whether permeability barrier has any part to play in not metabolizing such substrates is not known). Therefore, a lack of oxygen uptake may mean either (1) that these compounds are not further oxidised or (2) these compounds are not easily available for the cells to metabolize.

All the acidic fermentation products were, however, oxidised readily by limonene-grown cells (Fig. 31). This

indicates that the capacity for oxidation of these acids is inherent in limonene-grown cells. That this oxidation ability is not subsequently induced on these cells is shown by the fact that the rate of oxidation of these acids was not materially affected by the addition of chloramphinical.

The results in Table 15 and 16 (Fig.31 and 32) indicate that perillic acid is oxidised faster than the hydroxy or dicarboxylic acids. The rate of oxidation of dicarboxylic acid (68) by the limonene-grown cells is considerably slower than those of perillic and the hydroxy acids.

Preliminary experiments on the growth studies of the bacterium indicated that it grows profusely on glucose and sucrose. It was considered worthwhile to see whether glucose grown cells could oxidise limonene giving rise to some intermediate products.

In a typical fermentation, 24 hr-old limonene-grown cells were grown in the basal medium containing 1% glucose. After 24 hr growth on glucose medium, limonene was added and fermentation continued for 24 hr. The medium was extracted and processed in the usual manner, (Chapter II) and separated into acidic and neutral components.

The acidic fraction from the ether extract (A_1) was a solid (m.p. 120^{o}) which after recrystallization from

pet-ether melted at 124-125^{o} and was identical with synthetic perillic acid (66). There was no depression in the mixed m.p. and both the synthetic and the metabolite had an identical I.R. spectrum. The yields of perillic acid were about 50% on the basis of limonene added.

The neutral fraction was a gummy material. The crude product showed a strong band at 1680 cm^{-1} presumably indicating an unsaturated ester. The exocyclic methylenic double bond (890 cm^{-1}) was absent. The gummy material was hydrolysed with alkali and the neutral and the acidic components separated as usual.

The neutral component was chromatographed over alumina. The pet ether eluate consisted mainly of unreacted limonene and some amount of fat, while 10% ether eluate gave a monohydroxy compound (81) of the molecular formula $C_{10}H_{16}O$. The I.R. spectrum indicated the presence of a hydroxy group ν_{max} 3358, 1136, 1050 cm^{-1} indicating probably a primary hydroxy group and a methylenic and a trisubstituted double bonds (1645, 888, 812 cm^{-1}). The alcohol was found to be identical with the synthetic perillyl alcohol obtained by the reduction of perillic acid. Besides unreacted limonene, and a small quantity of perillyl alcohol, no other compounds could be characterized from the neutral fraction from the saponified compound.

The acidic components were converted into their methyl esters with diazomethane in ether. TLC showed the

presence of atleast three spots with approximate ratio 30:5:60. The methyl esters were subjected to chromatography over alumina.

The fraction eluting out with pet-ether was mainly of a fatty acid ester.

The pet-ether ether 5-25% eluate consisted mainly of a liquid, which was associated with a small quantity of a more polar fraction. Further purification was attempted by redistillation under reduced pressure. VPC of the distillate showed that the compound was about 80% pure. Further purification could not be achieved by rechromatography.

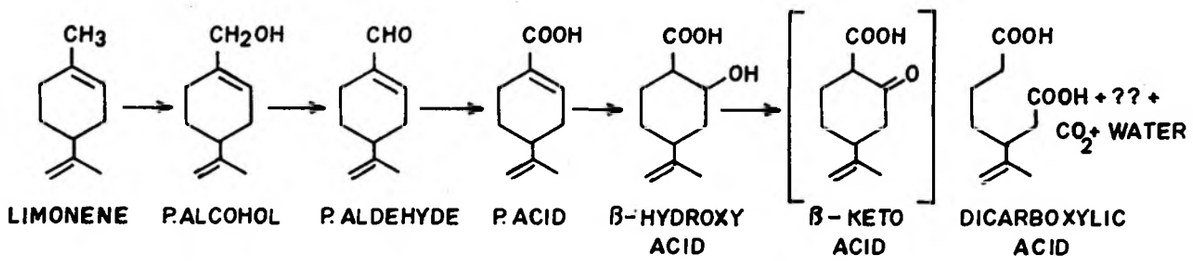
The fraction eluting out with 10%-70% ether consisted mainly of a single compound (VPC analysis indicated that it is 97% pure). Further purification of this compound was achieved by distillation under reduced pressure. The elemental analysis corresponded to an empirical formula $C_{11}H_{20}O_2$. I.R. spectrum indicated the presence of a hydroxyl group (I.R. γmax 3500, 1050 cm^{-1}) and an ester group (1732 cm^{-1}) which appeared to be nonconjugated. It did not exhibit any characteristic UV absorption. On acetylation with acetic anhydride and pyridine at room temp. it gave a mono-acetyl derivative (band at 1750 cm^{-1}). It was not possible to carry out further work on these acids due to paucity of material.

Sucrose-grown cells and molasses-grown cells behaved in a similar manner to that of glucose-grown cells yielding mainly perillic acid from limonene. The yields of perillic acid in these cases, however, were much lower.

It was not possible to separate any other acid from the mixture, besides traces of the dicarboxylic acid (68).

Probable pathway for limonene dissimilation

From the adaptive enzyme studies, one is tempted to presume that one of the major pathways of limonene degradation through the acidic compounds to carbon dioxide and water would be :



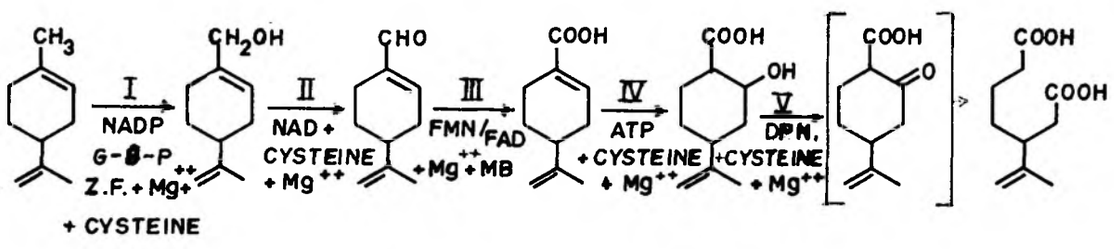
Further support for the proposed pathway for limonene oxidation came from the observation that on limonene fermentation by glucose-grown cells results in the formation of perillic acid as a major product along with small amounts of perillyl alcohol, both in the free as well as in the esterified stage.

One of the possible explanation which can be considered for the accumulation of perillic acid is the

possible enhancement of its rate of formation as compared to its rates of degradation. The acidic fraction also contained traces of dicarboxylic acid. This would indicate that there is no serious impairment of the mechanism of degradation of perillic acid in glucose-grown cells.

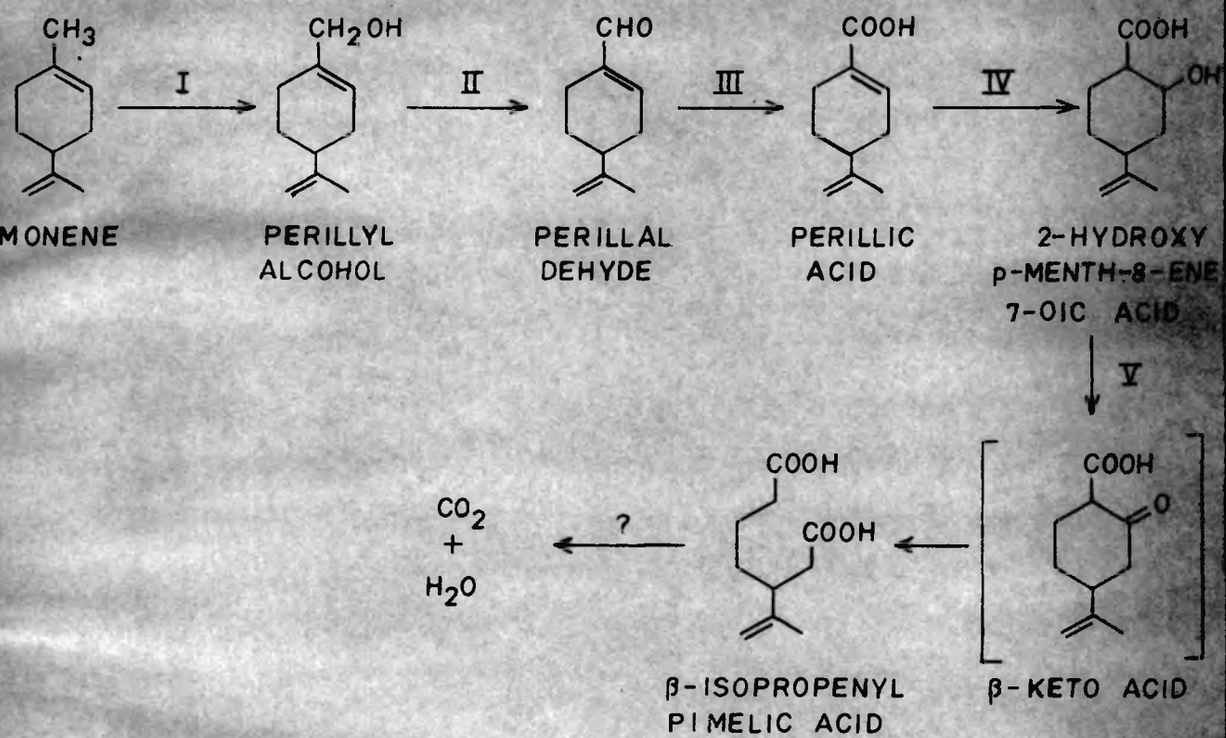
Perillic acid grown cells failed to oxidize limonene but surprisingly on the other hand did not show any oxygen uptake with dicarboxylic acid. In fact fermentation of perillic acid with perillic acid grown cells led to the accumulation of the dicarboxylic acid in the medium. It appears, therefore, that somehow growth on perillic acid impairs the activity of the enzymic sequence from the dicarboxylic acid to carbon dioxide and water.

When one postulates such a mechanism for the limonene degradation (chart 10, Pathway 1), it is possible to classify the enzymatic reactions involved on the basis of oxygenation, dehydrogenation and oxidation which in the presence of usual co-factors are expected to bring about the completion of the reactions. At least the adaptive enzyme studies helped in developing a working model as regards to what types of enzymes to look for. Preliminary enzymatic studies were conducted initially with the crude sonicates.



PATHWAYS FOR LIMONENE TRANSFORMATION

PATHWAY 1



PATHWAY 2

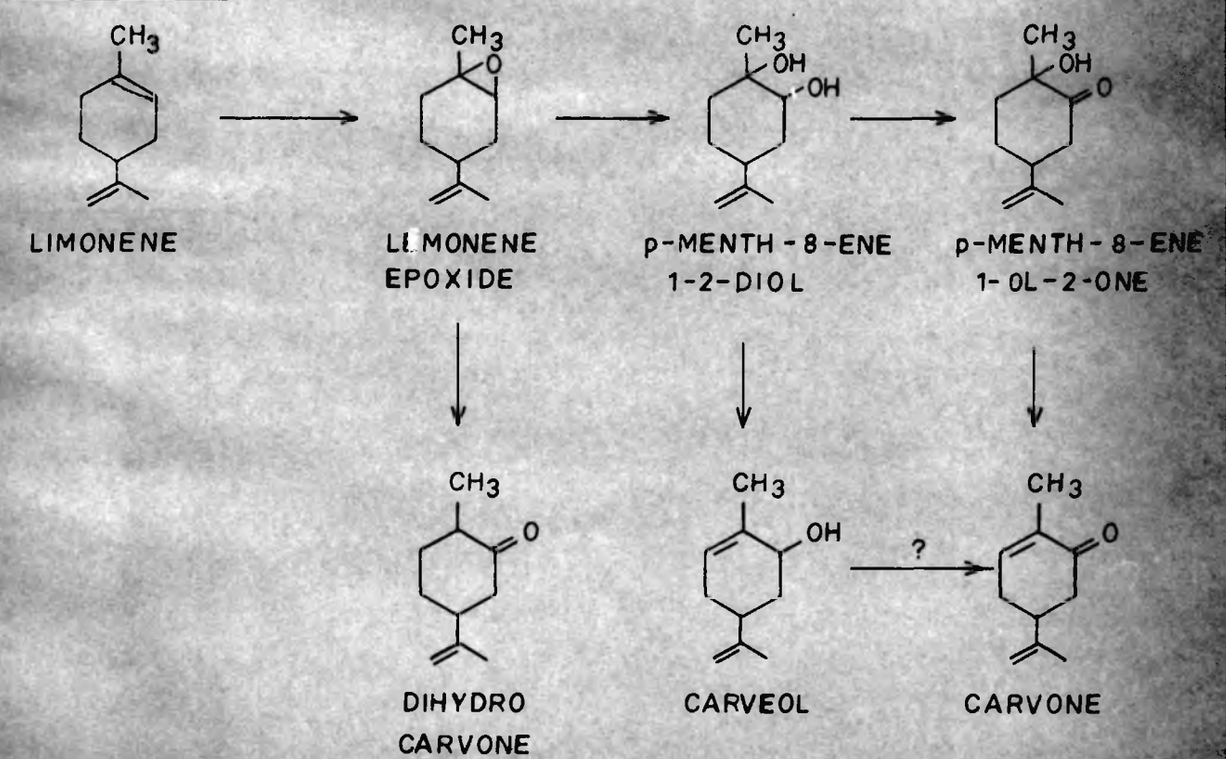


CHART – 10

The sonicates from limonene-grown cells did not show any oxygen uptake with any of the terpene substrates. This is not surprising, however, since it is known that the disruption of the bacterial cell usually bring about widespread destruction of pyridine nucleotides which may play a vital role in the oxidation process.

The enzymatic studies were monitored by two main techniques, thin-layer-chromatography and vapour-liquid chromatography of the products of the reaction. It was observed that with the unfractionated sonicates the first reaction was rather slow and little or none of perillyl alcohol (81) was formed. However incubation of limonene with NADPH along with a NADPH regenerating system consisting of Zwischenferment, glucose-6-phosphate and NADP resulted in the formation of perillic acid (66) along with other products indicating that the hydroxylation reaction might have possibly led to the formation of perillyl alcohol (81) and the alcohol was rapidly oxidised to perillic acid in the presence of NAD and perillaldehyde (82) with added FAD and FMN also gave rise to perillic acid (66) and traces of dicarboxylic acid (68).

In the test system for perillic acid (66), CoA-SH and ATP were added. An indirect evidence for the requirement of Co-ASH was deduced from the observation that neither limonene nor perillic acid-grown cells metabolised perillic

acid further under nitrogen atmosphere even after 24 hr. In this case ATP synthesis could have been seriously impaired under anaerobic conditions and so perillic acid could not be converted to the activated ester for further reaction.

Both perillic (66) and the hydroxy (73) acids gave rise to the dicarboxylic acid (68) with the cell free extract in the presence of ATP, CoA-SH, Mg^{++} and NAD.

Since an oxygenation reaction such as I is known to be carried out by particulate preparations, the sonicate was next fractionated to give particulate and supernatant fractions.

The cell free extract was subjected to ultra-centrifugation at 100,000 g for 1 hr. The supernatant was removed and the pellet was washed and recentrifuged. The particulate fraction was suspended in phosphate buffer and two sets of experimentswere conducted, one with the super-natant and the other with the particulate suspension. In order to concentrate the enzymes in the supernatant and to eliminate interfering metabolites and co-factors it was precipitated with ammonium-sulphate to 0.9 saturation. The ammonium sulphate precipitate was dissolved in a small amount of buffer and used for the enzyme studies.

It was observed, in the case of the experiments conducted with supernatant and with 0.9 saturation ammonium sulphate fraction of the supernatant, the reactions II to V

were proceeding smoothly. In reaction II, perillyl alcohol in the presence of NAD, cysteine, mg^{++} was converted to perillic acid. Traces of the dicarboxylic acid were also observed when the reaction products were monitored by TLC and VPC. (Plate I). The conversion of perillyl alcohol (81) to perillyl aldehyde (82) (i.e. reaction II) was not detected by TLC or by VPC, as the aldehyde was further oxidised to perillic acid. The fraction contained a powerful perillyl-aldehyde dehydrogenase activity and the aldehyde was converted to perillic acid and also traces of dicarboxylic acid in the presence of FAD/FMN + Mg^{++} with MB as an acceptor under anaerobic conditions. There was a complete disappearance of perillyl alcohol or perillyl aldehyde in the neutral fraction as evidenced by TLC or VPC. Perillic acid in the presence of CoASH, ATP, cysteine, and Mg^{++} ions was converted mostly to the dicarboxylic acid, β-hydroxy acid being formed only in traces. The hydroxy acid (73) in the presence of NAD, cysteine and Mg^{++} ions also gave rise to the dicarboxylic acid. (68). The intermediate i.e. keto acid (83) could not be detected by the above techniques.

Similar set of experiments were conducted using the particulate suspension. It was observed in this set of experiments that thefirstreaction is slow. The amount of conversion of limonene to perillyl alcohol in the presence of NADP, G-6-P, Zwishenferment Mg^{++} and cysteine was very small. When the reaction products were monitored by TLC it was observed that in addition to perillyl alcohol three

more feeble spots appeared. One of these could be identified as perillic acid. Apparently the particulate fraction contained some dehydrogenases causing further oxidation of perillyl alcohol to perillic acid.

Tables 17 and 18 gives the experimental details and the results obtained. It is interesting to note that the first steps in the degradation of limonene is more or less similar to that reported by Azoulay et al (1963) for that of n-heptane which is converted to heptylic acid by a soil pseudomonad.

It should be emphasised that no attempts were made to characterise any of the enzyme systems with respect to their co-factor requirements. The number of controls run were obviously inadequate for such characterisation studies. It should be noted that even in the 90% ammonium sulphate precipitate perillyl alcohol was converted to perillic acid inspite of the fact that DPN was the only co-factor added. It is possible that this fraction contained in the bound form the enzymes required for conversion of perillaldehyde to the acid.

In the present work experiments were designed just to qualitatively demonstrate the existence of all the enzymes leading to the dicarboxylic acid from limonene. This has been accomplished and the enzymatic data fully substantiate the conclusions derived for the adaptive enzyme studies.

The mechanism of formation of neutral compounds can be explained mechanistically by postulating an epoxidation on the 1,2 double bond of limonene. The epoxide (84) can give rise to dihydrocarvone (56) by a proton catalysed opening of the epoxide ring with a hydride shift. (Deno et al, 1960) and the diol (62) by the acid catalysed hydration. The trans disposition of the two hydroxy groups on the diol (62) appear to be consistent with the postulated mediation of epoxide as an intermediate. From the diol (62) it is easy to postulate the formation of carveol (58) by dehydration of the tert. hydroxyl group, and of the hydroxy ketone (60) by a simple oxidation. The formation of carvone (57) by elimination of the hydroxyl group in the hydroxy ketone can also be visualised to take place by accepted mechanisms. Alternative mechanisms can also be suggested (Chart-10)

It is noteworthy that carvone is formed in small amounts in the controls by an auto-oxidation process. Since the amount of this compound in the experimental flasks was considerably larger than that in the controls, it is tempting to conclude that the neutral oxygenated compounds are true transformation products. However, one needs caution in accepting such conclusions and the existence of pathway No.2 remains to be proven by adequate enzyme studies.

At this stage the relationship of the dihydroxy acid (79) with Pathway 1 remains obscure.

Experimental

(i) Manometric

Experiments were conducted with neutral and acidic transformation products with either limonene or glucose, sucrose or perillic acid grown cells. Twenty four hr grown cells were used in the manometric studies.

Cells grown on limonene (24 hr) were centrifuged, well washed with M/20 phosphate buffer and suspended in a phosphate buffer to give a turbidity reading of 200 at 660 mμ .

Oxidation of substrates by resting cells was followed by the conventional manometric procedure (Umbreit, Burries and Staufler, 1959). Each warburg flask contained (in a final volume of 3 ml) 3 to 10 mg of resting cells. 0.3 ml phosphate M/20 buffer 20 μ moles of substrate, and 0.2 ml of 20% KOH in the centre well. The concentration of chloroampheric ol when used was 50 μg. per ml.

Resting cells of cultures grown on limonene do not oxidise carvone, dihydrocarvone, diol 2, diol 3, perillyl alcohol, while perillic acid, hydroxy acids and dicarboxylic acid were oxidised at a comparable rate. (Fig.33).

The oxidation of perillic acid was rapid and was faster than that of the hydroxy acids and the dicarboxylic acid. Dicarboxylic acid was oxidised slowly as compared to the hydroxy acids and perillic acid.

.e (15). Oxidation of neutral and acidic transformation products of limonene by limonene-grown cells

Resting cells (24 hr old)

ɪth substrate	Qo_2^* µl/hr	
	Without CMP	With CMP
ɔnene	119	118
ɾone	0.5	0
ɾdrocarvone	0	-
. 2	+0.5	-
. 3	+1	+1
.llyl alcohol	0	-
.llic acid	86	87
ɾdroxy acid	30	31
ɪrboxylic acid	8	8
ɾdroxy acid	14	-

ɪese represent values after making correction for the ɪdogenous respiration.

Table (16). Oxygen uptake with neutral and acidic transformation products of limonene by glucose, sucrose and perillic acid-grown cells.

Growth substrate	Qo_2^* µl/hr
(a) Glucose-grown cells (24 hr old : Resting)	
Limonene	28
Diol 2	2
Carvone	0
Perillic acid	5
(b) Sucrose-grown cells (24 hr old : Resting)	
Limonene	23
Carvone	0
Perillic acid	12
(c) Perillic acid-grown cells (24 hr old : Resting)	
Limonene	8 **
Perillic acid	65
Dicarboxylic acid	4

* These represent values after making correction for the endogenous respiration.

** After a lag phase of 2 hr.

OXYGEN UPTAKE BY LIMONENE-GROWN CELLS WITH VARIOUS ACIDS

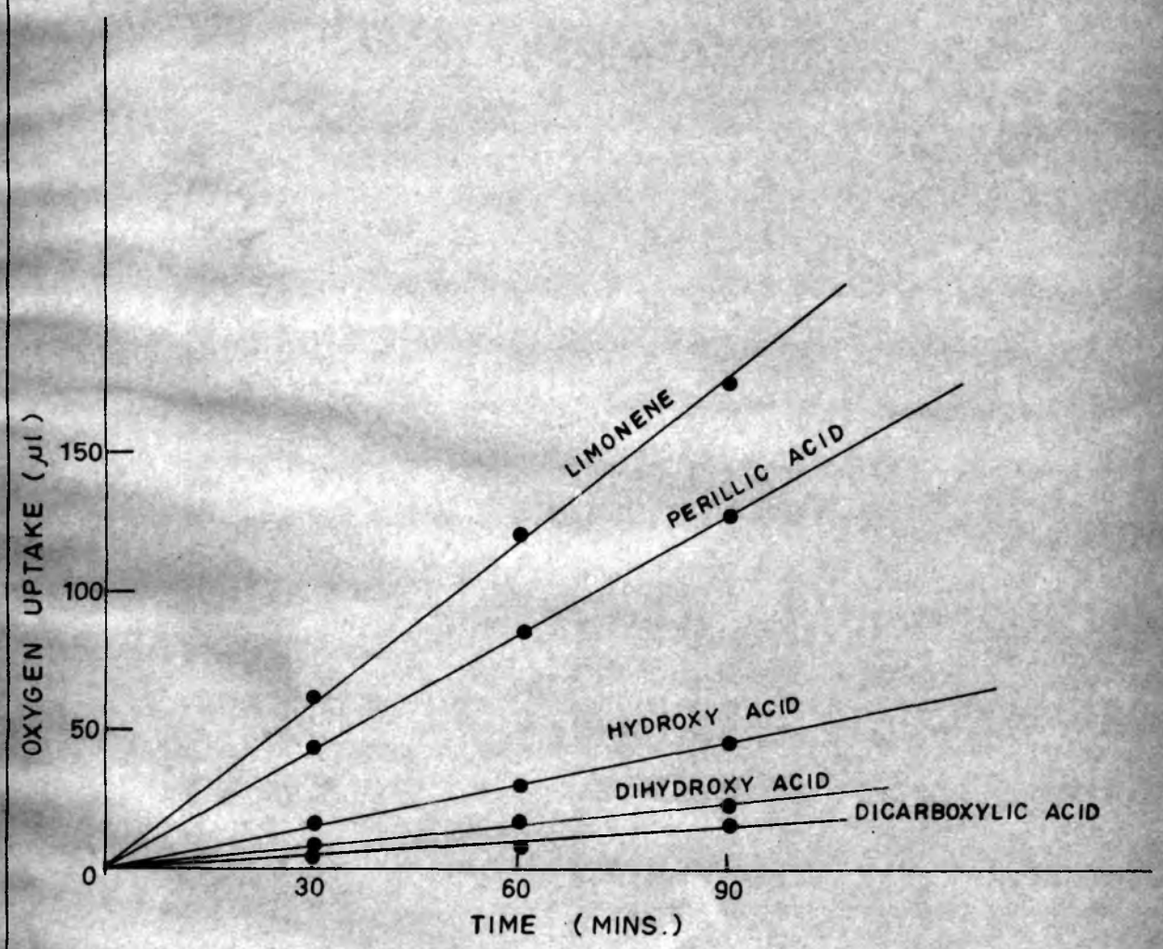


FIG.— 31.

OXYGEN UPTAKE WITH NEUTRAL AND ACIDIC TRANSFORMATION PRODUCT
OF LIMONENE BY GLUCOSE SUCROSE AND PERILLIC ACID GROWN CELLS

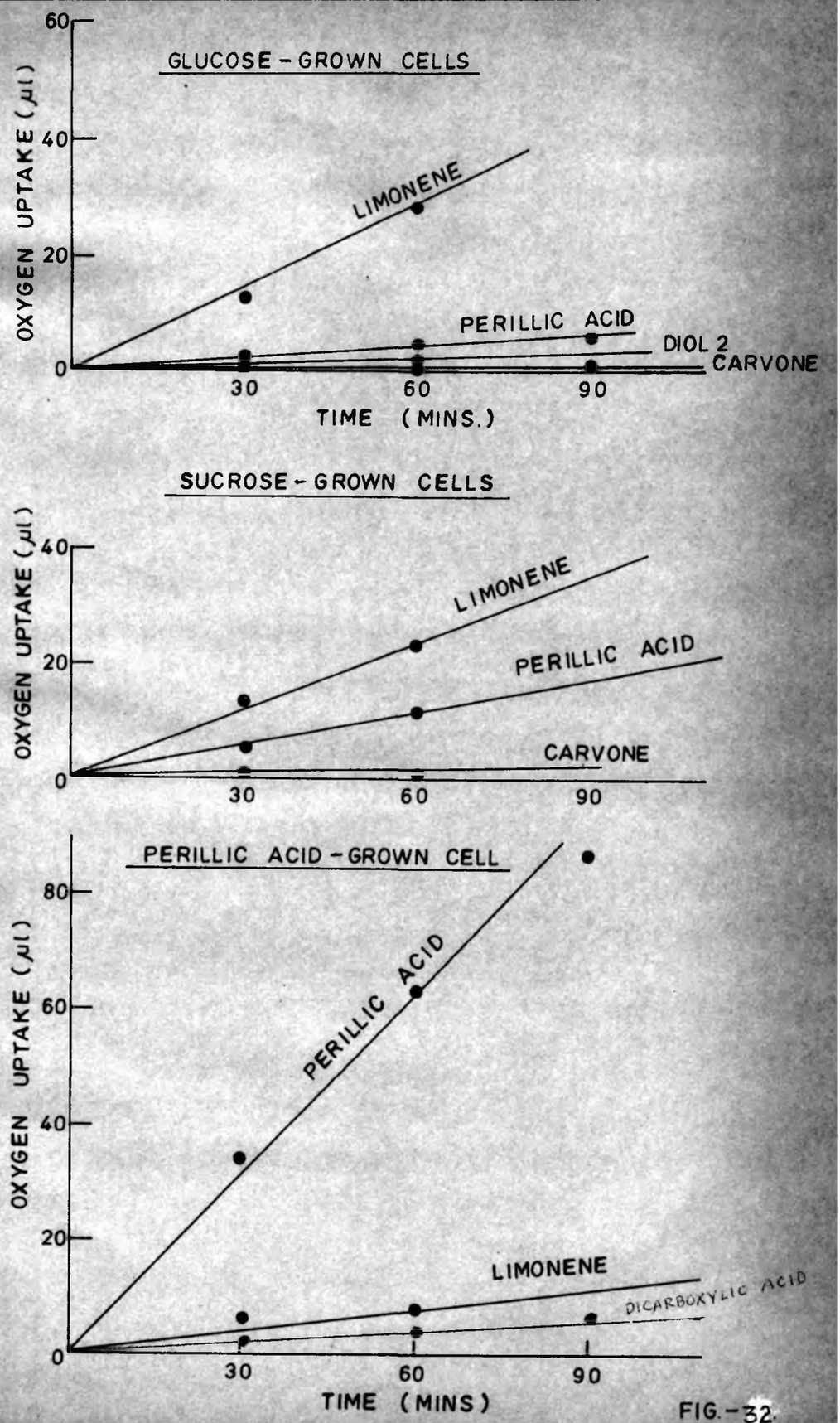


FIG. - 32.

(2) Fermentation with glucose-grown cells

Forty flasks each containing 90 ml basal medium were sterilized at 15 psi pressure for 20 minutes. To each of the flasks 10 ml of 10% sterile glucose solution was added. Limonene grown cells (24 hr old) were suspended in basal medium (turbidity 250 at 660 mμ, red filter) and added (2 ml inoculum to each flask) and the flasks incubated for twenty four hr on a rotary shaker (Temp. 28-30°). After 24 hr limonene (0.5 ml) was added to each flask and the fermentation continued for another 24 hr. At the end of the fermentation period (total 48 hr) the contents were pooled, acidified and extracted (500 ml x 3) with ether and finally with butanol (400 ml x 2) in the manner described in Chapter II - Materials and Methods. From the ether extract the neutral and acidic fractions were separated by washing with 5% sodium carbonate (50 ml x 3). The neutral fraction (5.85 g) was a gummy product. The crude product showed I.R. bands 1680, 1420, 1340 broad band between 1275-1050, 950, 712 cm^{-1}. The acidic product (A_1) (6.23 g) was a solid which after recrystallization from pet ether melted at 124-125°. I.R. absorption ν_{max} 2615, 1675, 1639, 1455, 1431, 1375, 1313, 1279, 1203, 1150, 1087, 1039, 955, 941, 927, 890, 863, 849, 799, 782, 739, 701 cm^{-1} (Fig. 16). (Found : C, 72.2%, H, 8.6%). The metabolite was characterized as perillic acid (66) through mixed m.p. determination and comparative I.R. spectroscopy.

Hydrolysis of the neutral fraction

To the neutral fraction (5.85 g) 40 ml 4N NaOH were added and the contents refluxed for 6 to 8 hr. The contents were then cooled, diluted with water (10 ml x 3) and extracted with ether (50 ml x 3). The sodium hydroxide extract after working up in the usual way gave the acidic fraction (2.8 gm). The neutral fraction (3.05 g) was subjected to chromatography separately.

Chromatography of neutral fraction

The neutral fraction (3.05 g) was chromatographed over alumina (60 gm) and the fractions eluted with pet ether, ether chloroform and methanol. The pet ether fraction gave unreacted limonene and some amount of fat.

Isolation of peryllyl alcohol (81)

The fraction eluting out with pet-ether-ether 10-50% gave a small quantity (120 mg) of liquid which after repeated distillation in vacuo (b.p. 85-95° bath, 2 mm) gave a monohydroxy compound. TLC on 15% ethyl acetate in hexane with perillyl alcohol as standard, the monohydroxy compound gave two spots one of which (major) had the same R_F value as perillyl alcohol. VPC showed 2 peaks, one corresponding to perillyl alcohol. Temp. 195°, 8 sec/10 ml 100:2 Poly Aze. 160 m.amp. 5 min.

Chromatography of the acidic components

The acidic fraction (2.8 g) was methylated with diazomethane in ether and the corresponding methyl esters (2.92 g) were subjected to chromatography over alumina (20 times the wt. of the material). Pet ether fraction eluted a semi-solid material (231 mg) which was identified as a fatty acid ester resulting from the bacterium.

Isolation of ester

The fraction (351 mg) eluted out with 5-10% ether in pet-ether was shown in TLC to be a single spot (ethyl acetate in hexane) 10%. R_F 0.80). VPC at 160°, 8.5 sec/10 ml and 150 m. amp. using poly ester as the stationery phase showed it to be a mixture of two components in which the major component (Ca 80%) ~~sample~~ had a retention time 7 min. I.R. absorption νmax 1730, 1662, 1460, 1938, 1320, 1275, 1200, 1188, 1120, 1037, 980, 910, 843, 820, 720 cm^{-1} (not shown).

Isolation of monohydroxy acid ester

The fraction eluted from the column in 10-75% ether was mainly of a single component (TLC single spot R_F 0.35, 10% ethyl acetate in hexane). Purified by distillation under reduced pressure b.p. 95-100°, bath 4 mm to a colourless liquid. Found : C, 65.98, H, 10.74%; I.R. bands νmax 3500, 1732, 1480, 1460, 1370, 1290, 1200,

1188, 1120, 1080, 1050, 1000, 932, 880, 850, 724 cm^{-1}. (not shown). VPC - retention time 6 min. 200°, 8.5 sec/ 10 ml, 150 m. amp. polyester.

Acetylation of methyl ester

To a small quantity of the methyl ester (55 mg) 0.2 ml pyridine and 0.5 ml acetic anhydride was added and kept intermittently shaken at room temp. for 24 hr. After working up the liquid acetyl compound (60 mg) was distilled b.p. 100-105° (bath) 5 mm. I.R. ν_{max} 1750, 1470, 1440, 1380, 1245, 1200, 1170, 1130, 1035, 950, 730 cm^{-1}. (not shown).

Fermentation of limonene by sucrose and molasses-grown cells

The condition for fermentation and extraction were essentially the same as those described for glucose-grown cells. The acidic fraction from the fermentation yielded perillic acid (10% yield). The yield of this acid was slightly lower (9%) with molasses grown cells.

[C] Fermentation of perillic acid

Ten flasks each containing 100 ml of basal medium were inoculated with a suspension of cells grown on perillic acid (1 ml, 200 at 660 mμ). Aliquots of 100 mg of perillic acid was added to each flask and the flasks incubated for 72 hours on a rotary shaker. Aliquots of fresh perillic acid (100 mg) were added to each flask after every 24 hours. After 72 hours of incubation, the contents were removed,

pooled and extracted with ether (50 ml x 3). The acidic and neutral components separated as usual (vide Chapter II - Materials and Methods).

Separation of solid methyl ester

The acidic fraction (200 mg) was converted into its methyl esters (210 mg) with diazomethane. A small amount of an ester (7 mg) appeared as a crystalline material from the reaction mixture. m.p. 108-110°. I.R. absorption ν_{max} 3450, 3300, 1725, 1600, 1450, 1412, 1370, 1210, 1080, 970, 840, 768, 692 cm^{-1}. UV absorption, λ max 226 mμ, ϵ 14000. Due to paucity of material no further work was possible on this compound.

Chromatography of the methyl esters

The ether soluble methyl esters showed four spots on TLC (15% ethyl acetate in hexane, R_F 0.75, 0.50, 0.40 and 0.3) in the approximate proportions of 15:60:15:5 corresponding to perillic (66), dicarboxylic (68) and hydroxy (77) acids when authentic samples were chromatographed side by side.

Separation of perillic, dicarboxylic and hydroxy acids

The methyl esters (200 mg) were chromatographed over alumina (8 g) and eluted with the usual solvent system (vide Chapter II - Materials and Methods).

The pet ether-ether (i.e. 5-10% ether) eluted a small quantity of liquid (~30 mg). The I.R. spectrum and retention time on VPC of this compound were identical with those of perillic acid methyl ester. The compound (67) (25 mg) was saponified with methanolic KOH (0.2 ml containing 4% KOH) and the reaction mixture kept at 25° overnight. It was then diluted with water (5 ml), acidified and then extracted with ether (5 ml x 3). On concentration the compound (66) crystallized out. On recrystallization with pet-ether it melted at 125°. Mixed m.p. was unchanged with an authentic sample of perillic acid.

The main fraction eluting with 10-50% ether consisted of a viscous liquid (125 mg) which after distillation under reduced pressure (b.p. 130-135° (bath), 0.2 mm) showed identical I.R. spectrum and retention time in VPC with those of the authentic sample of dicarboxylic acid methyl ester (69).

The last fraction eluted with 50-100% ether contained a mixture of hydroxy acids (30-40 mg) as revealed by TLC and VPC techniques. One of these corresponded to the hydroxy acid methyl ester (73).

Chromatography of the neutral fraction

The neutral fraction (75 mg) was chromatographed over alumina (2 g) and eluted with pet-ether and ether.

Pet-ether eluted a liquid (10 mg) b.p. 180-185° (bath), 2 mm, identified as bacterial fat.

The second fraction eluted with 10-50% ether was a liquid (50 mg). TLC showed two spots. Purification by distillation under reduced pressure, b.p. 80-85° bath/4 mm. I.R. ν_{max} 3400, 1625, 1475, 1450, 1378, 1338, 1315, 1170, 1150, 1125, 1090, 1040, 947, 926, 870, 840, 805 cm^{-1}. (Found : C, 44.35, H, 9.0%). No further work was possible due to paucity of material.

[D] Enzyme studies

The following substrates have been used in the present study. Limonene (55), perillyl alcohol (81), perillaldehyde (82), perillic acid (66), 2-hydroxy-p-menth-8-ene-7-oic acid (77) and β-isopropenyl pimelic acid (dicarboxylic acid) (68).

Of these perillyl alcohol and perillaldehyde were synthesised from perillic acid in the following manner.

Preparation of perillyl alcohol

Into a well dispersed ice-cold suspension of lithium aluminium hydride (400 mg) in anhydrous ether (20 ml) in an ice bath a solution of perillic acid (66) (300 mg) in ether was introduced with vigorous stirring. After stirring for 30 min in the ice bath, the reaction mixture was refluxed at 35° for 15 minutes. The mixture was then chilled in an ice bath and cautiously decomposed with ethanol--water (3:1) and then (3:2). The ether solution was decanted

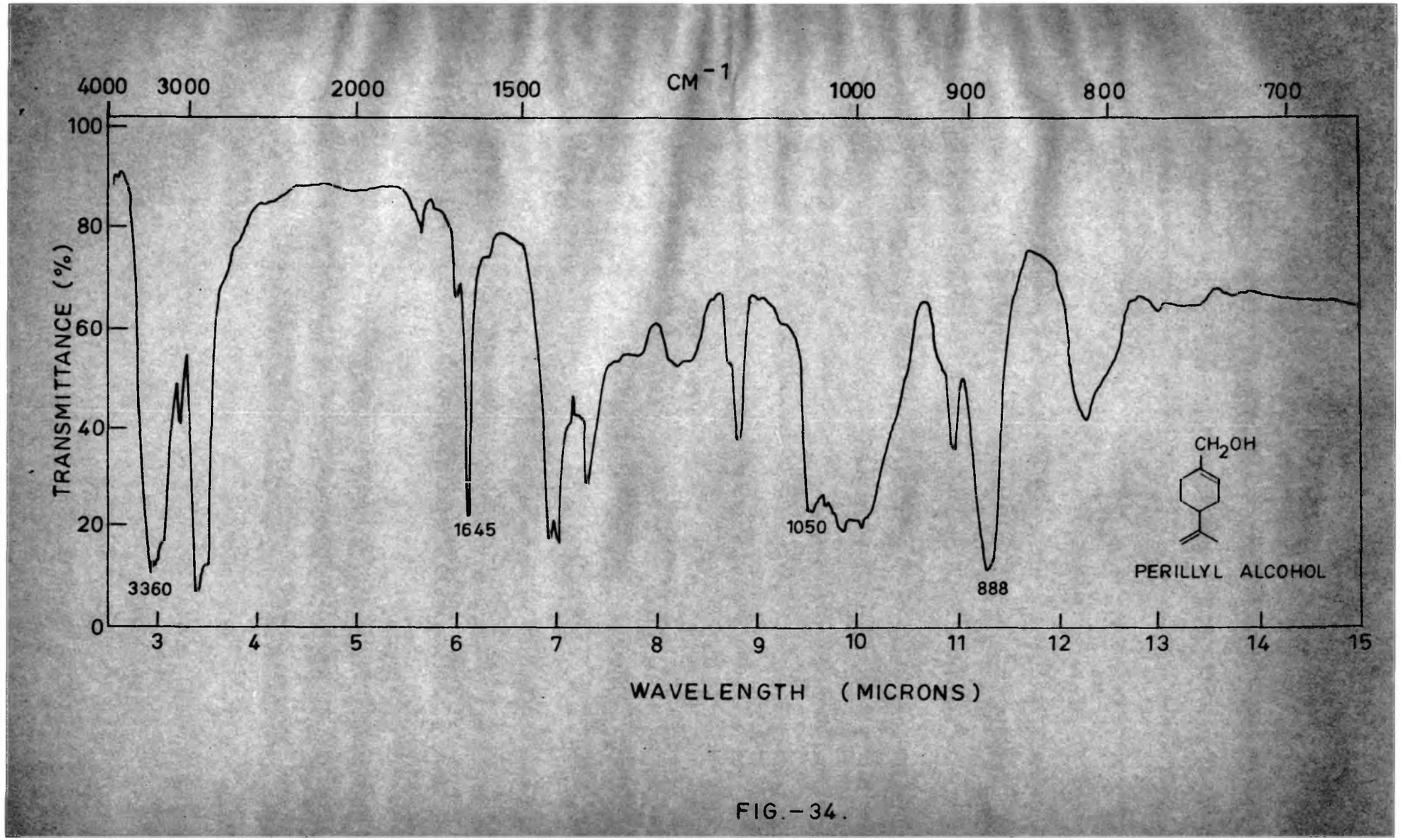

4000
3000
2000
1500
CM⁻¹
1000
900
800
700
100
80
60
40
20
0
TRANSMITTANCE (%)
3360
1645
1050
888
CH₂OH
PERILLYL ALCOHOL
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

FIG.-34.

off and the residue extracted with ether (10 ml x 3). The combined ether extracts were washed with saline, dried over sodium sulphate and concentrated. The product (230 mg) was distilled under reduced pressure b.p. 100-105° bath 4 mm, η_D^{30} 1.4920. I.R. ν_{max} 3360, 1645, 1450, 1435, 1370 1280, 1220, 1183, 1136, 1050, 1025, 1000, 915, 888, 812, 770 cm^{-1}. (Fig. 34).

Oxidation of perillyl alcohol to perillaldehyde (82)

Preparation of chromium trioxide in pyridine

To dry pyridine (2 ml) chromium trioxide (200 mg) was added with stirring. The ppt formed was filtered off and used for the experiment.

To perillyl alcohol (81) (200 mg) in 2 ml pyridine, chromium trioxide (200 mg) in pyridine (2 ml) was added with shaking. The contents were kept overnight. Next day after decomposition of excess of chromium trioxide with 5 drops of methanol, the mixture was diluted with 50 ml water and extracted with (25 ml x 2) ether. The ether extract was washed with water and then with a little 5% aqu. bicarbonate and finally with water. After evaporation a liquid (82) (160 mg) was obtained. b.p. 95-97° (bath), 4 mm. The I.R. spectrum of the compound corresponded to that of authentic sample.

Sonication of limonene-grown cells

Twelve flasks containing 100 ml basal medium were sterilized at 15 psi pressure for 20 min. Aliquots of 2 ml of a 24 hr limonene-grown cells suspension in M/20 buffer were added to each flask along with limonene (0.2 ml) and the flasks incubated on a rotary shaker for 24 hr (28-30°).

After 24 hr of incubation the cells were centrifuged for 1 hr at 20,000 g. The subsequent operations were carried out at 0°. The cells were washed with phosphate buffer (M/20), recentrifuged and were suspended in 40 ml M/20 phosphate buffer (5 g wet weight in 40 ml). The suspension was treated with 5 mg of nicotinamide (0.1 ml) and subjected to sonication (Raytheon magneto striction sonic oscillator - 10 kc, 250 w) for 15 min. The sonicate was centrifuged at 800 g. The supernatent (35 ml) was decanted and used for enzyme studies as unfractionated cell-free extract. Aliquots of the supernatent were further centrifuged at 100,000 g (37-38,000 RPM, 40 no. head) in a Beckman Model L ultracentrifuge for 30 min. The particulate precipitate (2 ml) was washed with phosphate buffer, recentrifuged and both the particulate fraction and the supernatant suspended separately in M/20 phosphate buffer (15 ml and 30 ml respectively) and used for the enzyme studies.

90% saturation-supernatant with ammonium sulphate

To 25 ml supernatant surrounded by ice, 21 g of

ammonium sulphate was added slowly with stirring. The time taken for the addition of ammonium sulphate was 30 min. The stirring continued for another 10 min and the suspension was centrifuged at 2500 RPM for 30 min. The precipitate was dissolved in 15 ml M/20 phosphate buffer and used for the enzyme studies. For boiled enzyme experiments the preparations were kept on a steam bath for 15 minutes.

Enzymatic reactions

Reactions in both the sets of experiments were carried out in pyrex boiling tubes. After the addition of substrates, cofactors and the enzyme suspension, the tubes were kept on a rotary shaker and the reaction mixture was incubated for 4 hr. The reaction was stopped by the addition of 6N HCl (1 ml) and ether (5 ml) to each tube and the contents in each tube (experimental, control and the boiled enzyme) were extracted with ether (5 ml x 3). The etherial layers after concentrating to a small volume (5 ml) were washed with 5% carbonate (3 ml x 2), to remove the acidic fraction. The neutral fraction (N_1) after working up (without purification) was taken up in ether (0.2 ml) and spotted on TLC. The 5% sodium carbonate layer after acidification with dil. HCl (6N) and extraction with ether (5 ml x 3) was converted into methyl esters (AMe) with diazomethane in ether. The methyl esters (A_1Me) were concentrated and without further purification were spotted on TLC. Standard neutral and acidic compounds were also

spotted along with the reaction mixtures. The chromatograms were developed in 15% ethyl acetate in hexane. The plates after spraying with concentrated sulphuric acid were heated in an oven at 120-150°. The spots of dicarboxylic acid methyl esters appeared only when the plates were heated to 140-150°. The plates 1 and 2 give the idea of the enzymatic reactions.

The ethereal extracts were also subjected to vapour liquid chromatography.

Table (17). Reaction : 1 : $CH_3 \longrightarrow CH_2OH$

Reaction mixture	Expt. (a)	Control (b)	Boiled enzyme (c)
Limonene	0.02 ml	-	+
TPN	0.1 ml, 0.1 M (0.1 μ mol)	+	+
G-6-P	0.1 ml, 0.1 M (20 μ mols)	+	+
Cysteine	0.1 ml (20 μ mols)	+	+
Zwischenferment	0.1 ml (16 units/ml)	+	+
Magnesium chloride	0.1 ml (10 μ mols)	+	+
Buffer pH 7.2 M/20	2 ml	+	+
Enzyme	1 ml	+	(boiled enzyme) 1 ml

Table (18). Reaction : 2 : $CH_2OH \longrightarrow CHO$

Reaction mixture	Expt. (a)	Control (b)	Boiled enzyme (c)
Perillyl alcohol	3-4 mgs	-	+
NAD	4 μ mols	+	+
Magnesium chloride	0.1 ml (10 μ mols)	+	+
Cysteine	0.1 ml (20 μ mols)	+	+
Buffer	2 ml	+	+
Enzyme	1 ml	+	(boiled enzyme) 1 ml

Table (19). Reaction : 3 : CHO ⟶ COOH

Reaction mixture	Expt. (a)	Control (b)	Boiled enzyme (c)
Perillaldehyde	2-5 μ mols	-	+
FAD + FMN	0.075 mg each in 0.05 ml	+	+
MB : M/5000	1 ml (side tube)	+	+
$MgCl_2$	0.1 ml (20 μ mols)	+	+
Enzyme	1 ml	+	(boiled enzyme) 1 ml
Buffer M/20	2 ml	+	+

Table (20). Reaction : 4 : $COOH \longrightarrow CH_2OH.COOH \longrightarrow (COOH)_2$

Reaction mixture	Expt. (a)	Control (b)	Boiled Enzyme (c)
Perillic acid	3 mgs	-	+
ATP	0.1 ml (10 µ mols)	+	+
CoA-SH	0.1 ml 2 µ mols	+	+
+ NAD	10 µ mols	+	+
Cysteine	0.1 ml	+	+
	(10 µ mols)	+	+
$MgCl_2$	0.1 ml	+	+
	(10 µ mols)		
Buffer M/20	2 ml	+	+
Enzyme	1 ml	+	(boiled enzyme) 1 ml

Table (20_a) : TLC of supernatant

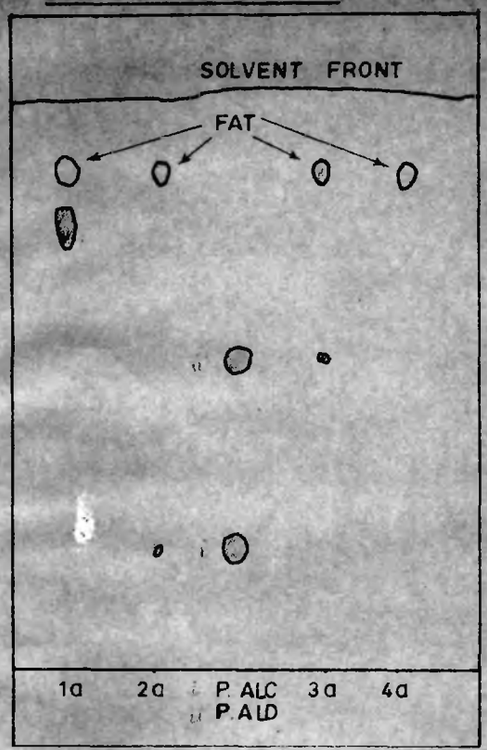
Substrate	Expt. (a)	Control (b)	Boiled Enzyme (c)	Remarks
1. $CH_3 \rightarrow CH_2OH$		-	-	Reaction seems to be very slow or absent
2. $CH_2OH \rightarrow CHO$		-	-	Reaction proceeds rapidly. Perillic and traces of dicarboxylic acid formed.
3. $CHO \rightarrow COOH$	very powerful	-	-	Reaction proceeds very rapidly. Perillic acid and small quantity of dicarboxylic acid are formed.
4. $COOH \rightarrow {}^{R}CHOH-$ $-COOH \rightarrow (COOH)_2$		-	-	Reaction is quite rapid with the accumulation of dicarboxylic acid and small quantity of hydroxy acid.
Pellets (Particulate) :				
The procedure is similar to that of in sonicate				
1. $CH_3 \rightarrow CH_2OH$		-	-	Reaction proceeds slowly.
2. $CH_2OH \rightarrow CHO$		-	-	Reaction proceeds to some extent. Traces of perillic acid is formed.
3. $CHO \rightarrow COOH$		-	-	Perillic acid and dicarboxylic acid seem to be formed in traces.
4. $COOH \rightarrow (COOH)_2$		-	-	Dicarboxylic acid in traces is formed.

Table (20)b. Retention time of the neutral and acidic components in VPC

Component	Column temperature	Retention time	Column
Perillyl alcohol	160°	17-19 min	
Perillyl alcohol	195°	4-5 "	Polyester
Perillic acid methyl ester	195°	4 "	succinic acid
Dicarboxylic acid	195°	7 "	
Hydroxy acid methyl ester	195°	10-11 "	

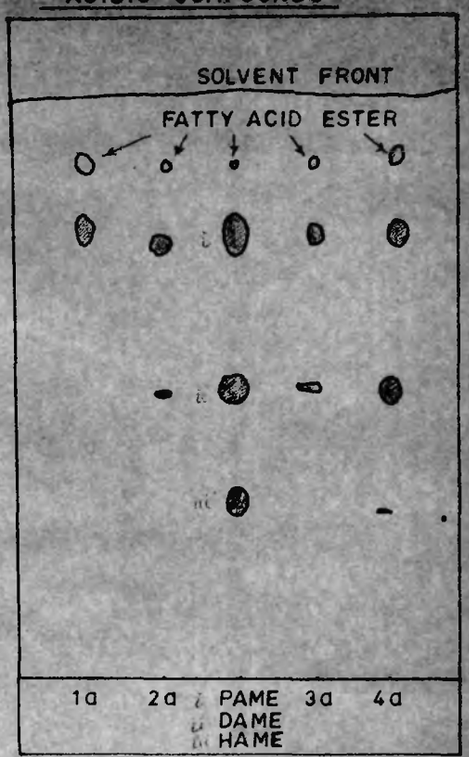
SONICATE (SUPERNATANT)

NEUTRAL COMPOUNDS



P. ALC – PERILLIC ALCOHOL
P. ALD – PERILLALDEHYDE

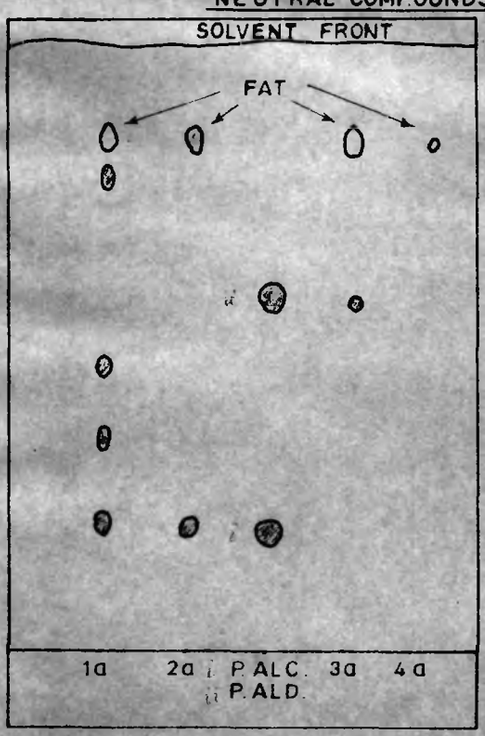
ACIDIC COMPOUNDS



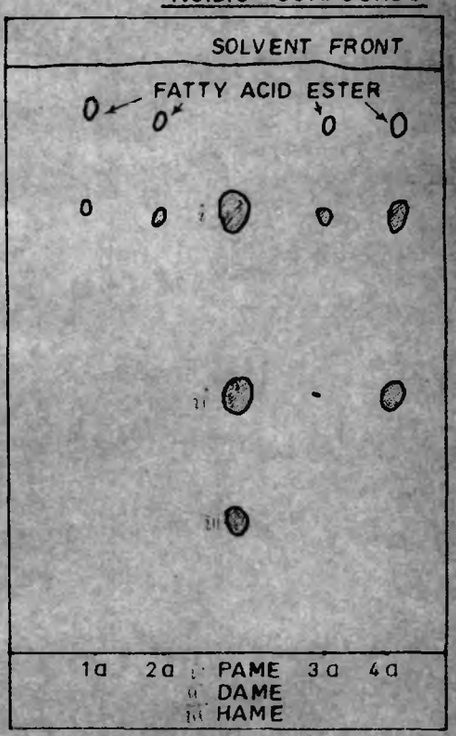
PAME – PERILLIC ACID METHYL ESTER
DAME – DICARBOXYLIC ACID METHYL ESTER
HAME – HYDROXY ACID METHYL ESTER

PELLET

NEUTRAL COMPOUNDS



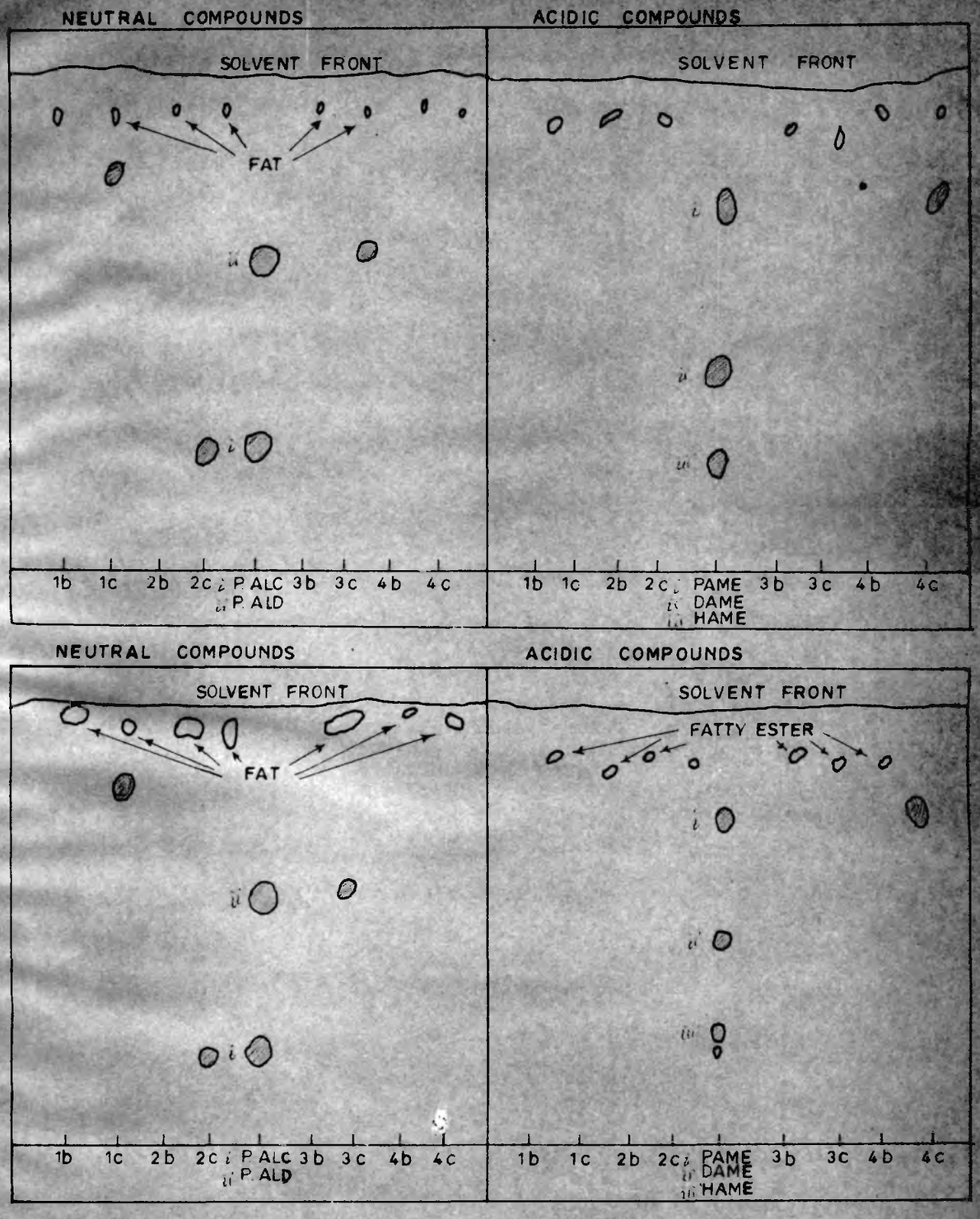
ACIDIC COMPOUNDS



SOLVENT : 15 % ETHYL ACETATE IN HEXANE.

PLATE - 1.

SONICATE (SUPERNATANT)



SOLVENT – 15% ETHYL ACETATE IN HEXANE

P.ALC – PERILLYL ALCOHOL
P.ALD – PERILLALDEHYDE

PAME – PERILLIC ACID METHYL ESTER
DAME – DICARBOXYLIC ACID METHYL ESTER
HAME – HYDROXY ACID METHYL ESTER

PLATE – 2.

CHAPTER - VII

S U M M A R Y

SUMMARY

Extensive studies on the microbiological transformations of steroids have been made for a decade. The interest in the microbiological transformations of terpenes, however, is of recent origin.

(1) Terpene transformations by fungi

In the present work it has been shown that 2, nonene, 2,3-dicarboxylic acid anhydride [which was previously isolated by Prema (1962) by incubation of camphene and β-santalene using A.niger] can also be obtained by the action of A.niger on longifolene. The rate of accumulation of the anhydride followed closely that of the disappearance of longifolene. Further degradative and synthetic studies which confirm the structure for the anhydride are presented.

It was also found that fermentation of two other hydrocarbons, caryophyllene and δ-cadinene also result in the formation of the anhydride. Furthermore, it was possible to detect small amounts of the anhydride by paper chromatography in control experiments run without terpenoid hydrocarbons. These results indicate that the anhydride may not be derived from terpenoid hydrocarbons. The probable mode of its origin is discussed. Tracer studies have indicated that this anhydride appears to have originated from the condensation of octanoate or its equivalent and pyruvate.

Fermentation of β-pinene by the experimental strain of A.niger 612 resulted in the formation of β-terpineol.

(2) Terpene transformations by bacteria

A soil pseudomonad capable of growing on limonene as the sole source of carbon was isolated by enrichment culture techniques. Preliminary studies on the organism with respect to its growth and nutritional requirements were made.

Fermentation of limonene by this bacterium resulted in the formation of a large number of neutral and acidic transformation products in small quantities. These products were separated into various individual components by using techniques such as solvent-solvent-distribution, chromatography and fractional vacuum distillation. The separation and isolation of these compounds were monitored by thin-layer chromatography as well as vapour-liquid partition chromatography

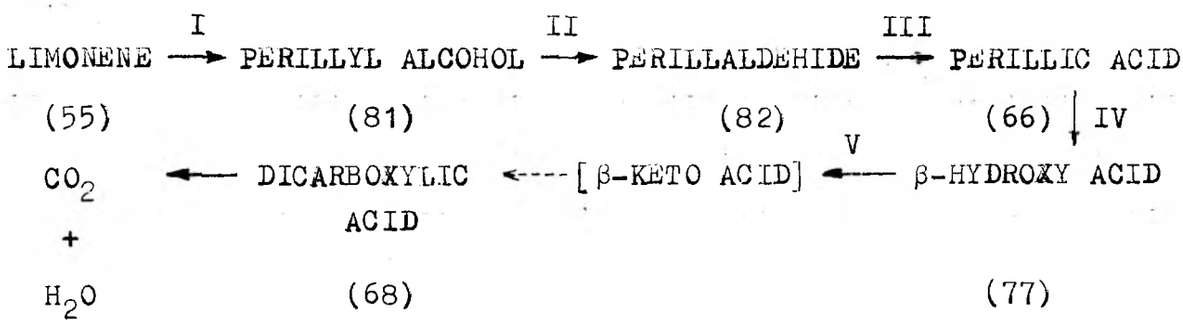
Five of the neutral compounds were identified as (1) dihydrocarvone (2) carvone (3) carveol (4) p-menth-8-ene 1-ol-2-one and (5) p-menth-8-ene-1,2, diol. From the acidic fractions the following were isolated and identified :- (6) perillic acid (7) 2-hydroxy-p-menth-8-ene 7-oic acid and (8) β-isopropenyl-pimelic acid. In addition to the above compounds several others could be isolated from the fermentation mixture but in quantities insufficient for complete characterization.

(3) Enzymatic studies

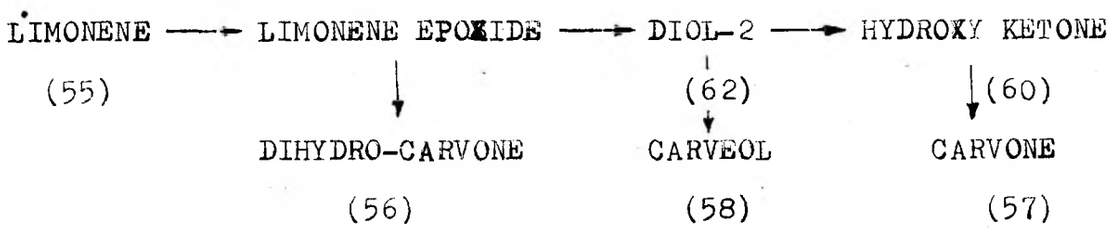
Growth and adaptive enzyme studies indicated that

none of the neutral compounds 56 to 62 is on the direct pathway of oxidation of limonene. The acidic compounds 66, 77 and 68 are all oxidised by limonene-grown cells. Limonene added to cells grown on glucose is converted mainly to perillic acid, and in small amounts to perillyl alcohol. Perillic acid grown cells accumulate isopropenyl pimelic acid. Based on these data the oxidation of limonene could be explained on the basis of two main pathways :

PATHWAY 1.



PATHWAY 2.



The unfractionated cell-free sonicates from the limonene-grown cells carry out the entire sequence of reactions I to V in the presence of added cofactors such as NADPH, NAD, FAD, FMN and ATP, cysteine and Mg^{++} ions. It has been shown that the first reaction in pathway I, the

hydroxylation of limonene in the presence of NADPH and oxygen is carried out by the sediment obtained on centrifugation of the cell-free extract at 100,000 g. The supernatant fraction from the 100,000 g sediment was precipitated at 0.9 saturation ammonium sulphate at 0°. The precipitate obtained was shown to convert perillyl alcohol to perillic acid with NAD and to transform perillaldehyde to perillic acid in the presence of methylene blue, FAD or FMN. It also converted perillic acid to the dicarboxylic acid, β-isopropenyl-pimelic acid, in the presence of ATP, CoASH and NAD. The enzyme activities were monitored by thin layer chromatography and vapour-liquid-partition chromatography.

The enzymatic evidence was consistent with the proposed pathway for limonene degradation.

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B I B L I O G R A P H Y

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