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**MICROBIOLOGICAL TRANSFORMATIONS
OF
TERPENES**



**A THESIS
submitted to the
UNIVERSITY OF POONA
for the degree of
DOCTOR OF PHILOSOPHY**

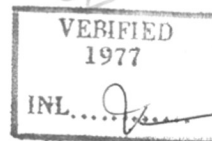
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List of abbreviations

I.R. : Infra red
L.A.H.: Lithium aluminium hydride
N.M.R.: Nuclear magnetic resonance
P.P.A.: Polyphosphoric acid
T.L.C.: Thin layer chromatogram
U.V. : Ultraviolet
V.P.C.: Vapour phase chromatogram
ATP : Adenosine triphosphate
CoA : Coenzyme A
CMP : Chloramphenicol
DNP : 2,4-Dinitrophenol
FAD : Flavin adenine dinucleotide
FMN : Flavin mononucleotide
MB : Methylene blue
NAD : Nicotinamide-adenine dinucleotide
NADH : Reduced form of NAD
NADP : Nicotinamide-adenine dinucleotide phosphate
NADPH : Reduced form of NADP

CHAPTER I
INTRODUCTION

INTRODUCTION

The biological degradation of natural compounds constitutes an important step in the carbon cycle of nature. A considerable amount of information is available today on the biotransformations of various classes of compounds such as steroids, alkaloids, aliphatic and aromatic compounds, including hydrocarbons, carbohydrates and antibiotics etc. (Tamm 1962, Pasqualini 1963, Evans 1963, Fuhs 1961, Dagley et al 1960, Foster 1962).

The aim of these studies is varied, ranging from the production of useful compounds such as the production of alcohols, acids, vitamins, amino acids and antibiotics through the agency of microorganisms, to the mechanism of microbial degradation of various insecticides, preservatives and detergents etc.

The field of microbial conversions achieved great stimulus from the work of Peterson & Murray (1952) on the hydroxylation of steroids by certain fungi leading to a new way of synthesis of a number of steroidal hormones such as cortisone, cortisol, aldosterone, etc. Since this subject has been reviewed extensively (Pasqualini 1963, Fried et al 1955, Epstein et al 1956, Talalay 1957, Vischer and Wettstein 1957 and 1958, Tamm 1960) it is not necessary to discuss it in detail here.

The first report on the degradation of paraffins by Botrytis cinerea appeared about 70 years ago (Miyoshi 1895). However, only recently, a considerable interest in this field has been

apparent with the realisation that the hydrocarbon substrates could be among the cheapest potential sources of proteins, amino acids, fatty acids and other useful products (Zobell 1946, 1950, Johnson 1964, Miller et al 1964, Darlington 1964, Shio et al 1963, Yamada et al 1963). By the use of alkanes as sole carbon source, a number of workers have obtained high cell yields of *Nocardia* and some yeasts (Champagnat 1962, 1963, Anon 1964, Darlington, 1964, Johnson 1964, Miller et al 1964, Raymond and Davis 1960, Fermentation Rev.No. - Biotech. & Bioeng. VI (1964)-57). Products from these organisms may find application as useful feed substitutes in future.

Microbiological transformation of terpenes

Earlier observation: The chemistry of terpenes has undergone development very rapidly, since they are of great value in perfumery industry and used to a smaller extent in the pharmaceutical products. Until recently the interest in the microbiological transformation of terpenes was sporadic and rather disconnected. The first report is by Myer and Neuberg (1915) who obtained citronellol in 59 per cent yield by reduction of citronellal using bottom yeast. Neuberg and Korb (1918) later obtained the conversion of citral to geraniol in about 30 per cent yield using yeast as the test organism. Cyclocitral was scarcely attacked by this yeast. Molineri (1929) achieved the conversion of citronellal to the corresponding alcohol and acid with the help of *Acetobacter xylium* and *A. nascentans*. *Penicillium digitatum* was found to effect the conversion of pulegol, isopulegol and citronellal to menthol in high yields ranging upto 93 per cent (Babicka et al 1955). Other known examples of such

transformations are the conversion of coniferyl alcohol (Pauly & Feuerstein, 1929) and a phytochemical reduction of 2,3 diketocamphane to 3 hydroxy camphor by bakers yeast (Neuberg, 1950). Dextrocamphoroquinone gave d-hydroxy camphor while the racemic compound gave a leavorotatory hydroxy compound.

Microbial degradation of camphor

The first systematic study on the microbial degradation of a terpenoid compound, camphor, began a few years ago at the University of Illinois (Bradshaw et al 1959). In the last six years several strains of bacteria have been isolated metabolising this compound as the sole source of carbon. Of these, two strains *Pseudomonas* C₁ and C₅ (Hedegaard et al 1961), degrade camphor by the same pathway but differ in their ability to use this compound as the sole source of carbon. The pattern of accumulating products also shows some quantitative difference. Besides these pseudomonads, a Diphtheroid Strain, T₁, also metabolises camphor but by a different pathway.

The 2-5 diketocamphane pathway

Fermentation of D(+) camphor (1) by pseudomonad C₁ results in the accumulation of a number of neutral and acidic products among which 2,5 diketocamphane (2) 5-exohydroxycamphor (3) 5-endohydroxy camphor (4), and 3,4,4 trimethyl-5-carboxymethyl²cyclopentenone (5) have been identified. The complete oxidation of this acid (5) was inhibited by 2,2' bipyridine with the accumulation of a lactic acid (6) (Fig.1). The pathway for the degradation of camphor was postulated as follows:



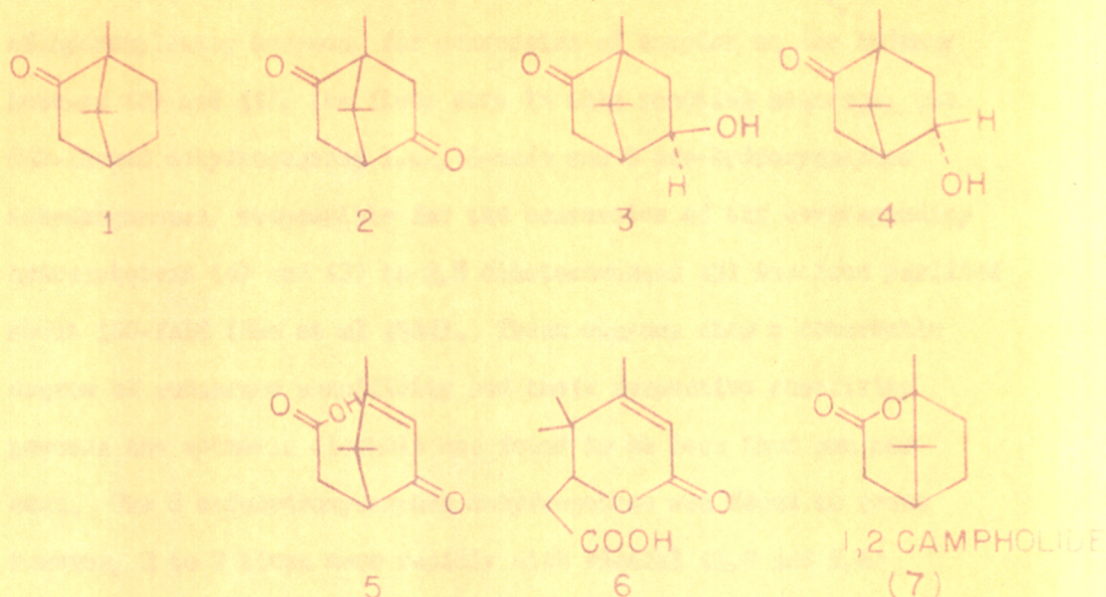


FIG I INTERMEDIATES IN CAMPHOR METABOLISM BY A PSEUDOMONAS

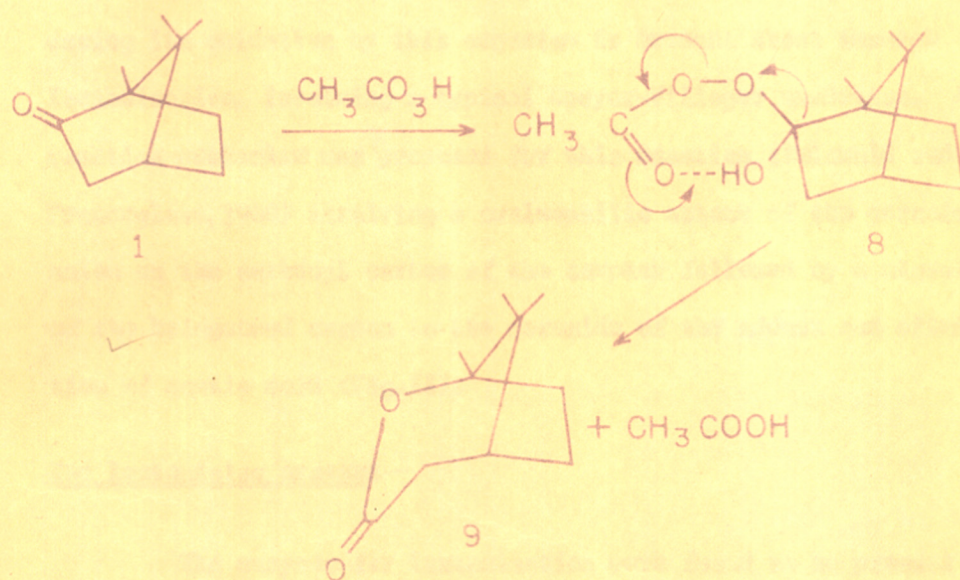


FIG. II BAEYER — VILLAGER OXIDATION OF CAMPHOR

While no information seems to be available about the nature of hydroxylating enzymes, for conversion of camphor to the hydroxy ketones (3) and (4), the first step in this reaction sequence, two NAD-linked dehydrogenases i.e., 5-endo and 5-exo-hydroxycamphor dehydrogenases, responsible for the conversion of the corresponding hydroxyketone (4) and (3) to 2,5 diketocamphane (2) have been purified about 100-fold (Kuo et al 1964). These enzymes show a remarkable degree of substrate specificity and their respective reactivity towards the epimeric alcohols was found to be less than one per cent. The 5 endohydroxycamphor dehydrogenase was found to react however, 2 to 3 times more rapidly with vicinal (2,3 and 5,6) diketones than with its natural substrate, 2,5-bisnaredione.

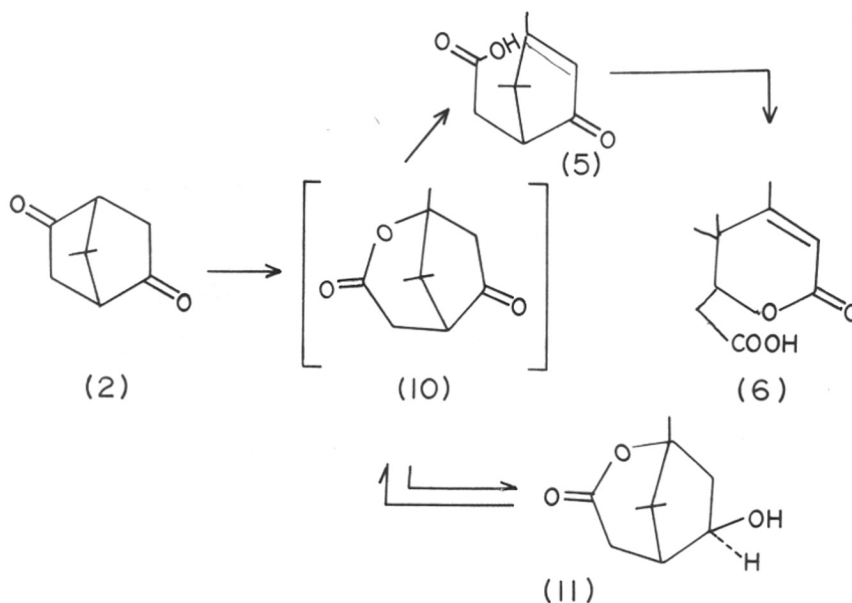
The cleavage of both the carbocyclic rings of camphor during its oxidation by this organism is brought about through lactonisation, involving a typical Baeyer-Villiger oxidation. A reaction mechanism was proposed for this reaction (Mainwald and Frauenglass, 1960) involving a nucleophilic attack of the peracid anion on the carbonyl carbon of the camphor followed by a migration of the bridgehead carbon to the peroxide of the adduct and elimination of acetic acid (Fig.II).

The lactonising systems—

The enzymes for lactonisation were found to be present in cell-free extracts (Conrad et al, 1961a), which converted camphor to 1,2 camphyllide (7) and 2,5 diketocamphane to 3,4,4-trimethyl-5 carboxymethyl- Δ^2 -cyclopentenone (5) in the presence of added NADH and oxygen. In dialysed extracts the need for FMN was also demonstrated. Bipyridine treatment was found to inactivate this

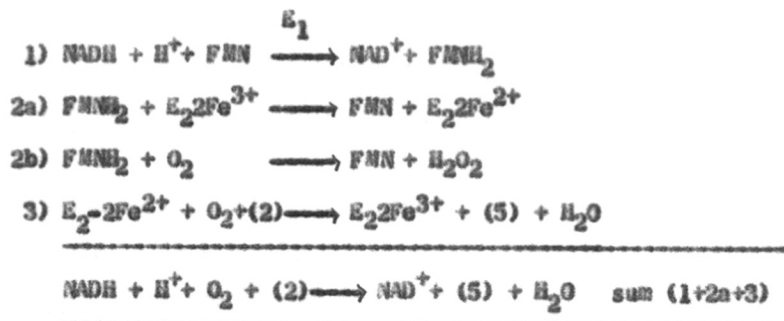
enzyme system which could be reactivated by Fe^{++} ions. Conrad et al (1961b) proposed the following reaction sequence for the conversion of 2,5 diketocamphane to (5) (Fig.III). The formation of (5) from (2) was proposed to take place through an unstable intermediate (10).

FIG. III



In addition to (5), (11) was also obtained as a side product. The unfractionated extracts also contained a dehydrogenase responsible for conversion of (11) to (5) again via the intermediate (10). On further investigation the lactonising system revealed some interesting features. Two different enzyme fractions are needed for this reaction in addition to NADH, FMN and Fe^{++} . The first enzyme (E_1) is a FMN-reductase and provides electrons whereas the second enzyme (E_2 -lactonising enzyme) undergoes oxidation reduction and reacts

with substrate as follows:



The first enzyme in this reaction sequence, the FMN coupled NADH oxidase has been purified about 100-fold (Bertland et al, 1963). Enzyme induction studies have demonstrated that cells growing on succinate, malate or glucose start synthesizing the oxidase on addition of the inducer. The rate of enzyme formation appears to be a function of the nature of the inducer and the primary carbon source. The formation of a high concentration of oxidase protein takes place only after the exhaustion of the primary sources. 2,5-Diketocamphane (2) was found to be the most potent inducer followed in decreasing order by campher and compound (5).

The lactonising enzyme itself has also been purified about 50-fold from campher-induced cells and behaves as a single component on ultracentrifugation and starch gel electrophoresis (Conrad et al 1962). For the lactonisation reaction FMN at 10^{-5}M concentration has the maximal activity, FAD being one third as active and riboflavin showing no activity at all. The inactivation of the reaction by 10^{-3}M bipyridine can be completely reversed specifically by 10^{-4}M Fe^{2+} , other metals being inactive. Neither of the two enzyme fractions by themselves reacts with bipyridine in the oxidized form

7

but after reduction of E_2Fe with FMN reductase system or chemically with sodium dithionite the enzyme bound Fe^{2+} does complex with bipyridine. Other reducing agents such as cysteine and glutathione are incapable of reducing this enzyme-bound iron.

The molecular weights of both the enzyme fractions, E_1 and E_2 , have been estimated to be about 50,000 and 80,000 respectively, by sucrose density-gradient centrifugation. Enzyme, E_1 , is devoid of cofactors. E_2 , however, contains significant levels of bound iron and flavin (Gunsalus et al 1964). Heme, though present in the early stages of purification (Conrad et al 1962), is reduced to very low levels in pure E_2 preparations. Inhibition studies with carbon monoxide, cyanide and azide indicate that this heme does not play any role in lactonisation.

The rate of NADH oxidation with E_1 , if measured in the absence of campher but with an excess of added FMN, is found to be considerably higher. Since the FMN concentration in the extracts is already sufficient to saturate the oxidase, it has been concluded that the flavin in these extracts is not autooxidisable. Both the enzymes are considered to exist in some sort of loose complex which makes flavin poorly accessible to oxygen, thus limiting the rate of autooxidation. On addition of the substrate there is a stimulation of NADH oxidation due to a rapid flow of electrons through the system via the lactonisation mechanism and not by $FMNH_2$ autooxidation.

The inhibitor of lactonisation, dipyridyl, on the other hand, was found to stimulate the oxygen uptake in the absence of any added substrate probably by chelating the E_2 -bound Fe^{2+} in such a way that autooxidation of $FMNH_2$ becomes possible. The amount

of this stimulation is equivalent to the substrate stimulation and cannot be further enhanced by the addition of camphor.

Centrifugation studies, on the other hand, demonstrated that in crude extracts both the E_1 - and E_2 -proteins sediment at the rates of pure preparations. Thus, if any physical coupling of the two enzymes is involved, it must be of a low order.

The stoichiometric relation of the reactions catalysed by this complex (One mole of NADH oxidation, O_2 consumption, substrate utilisation and product accumulation) has been demonstrated in fractionated extracts (Conrad et al 1964_a, Gunsalus et al 1964). The electron carrier sequence in this complex is as follows.



The purified lactonising complex is capable of catalysing the lactonisation of camphor, 2,5 diketo camphane, 5-exo and 5-endo hydroxycamphor, although with widely different K_m 's and turnover rates. With the help of mixed substrates it has been demonstrated that all the compounds react with the same active centre.

The lactonisation catalysed by this complex is inhibited by several group of compounds (Conrad et al 1964_b, Gunsalus et al 1964). Chelating agents block the passage of electrons to O_2 by coordination of Fe^{2+} in E_2 . Electron acceptors like methylene blue, 2,6 dichlorophenolindophenol and high concentrations of FMN intercept electrons from the FMN bound in complex. Metal ions appear to inhibit E_1 -catalysed transfer of electrons from NADH to FMN. A number of keto acids also inhibit the lactonisation reaction probably as alternative substrates. Peroxidase substrates such as ascorbate, catechol, etc. seem to block the normal product formation by

destruction of E_2Fe^{2+} -bound peroxide. Hydrogen peroxide does not take part in lactonisation and it has been suggested that the actual lactonisation is brought about by some sort of an enzyme-bound peroxide or an enzymic peracid formed by interaction of this peroxide with the carboxyl group of an adjacent acidic amino acid (Gunsalus et al 1964).

The lactonisation reaction has been classified with the mixed function oxidases which are also involved in the hydroxylation and lactonisation of steroids by microorganisms. Jacobson et al (1964) found that in addition to *Pseudomonas* C_1 , another strain of *E. testasteroni*, is capable of utilising camphor as the sole source of carbon. The induction of both the enzymes E_1 and E_2 can be brought about by either camphor or 17-ketosteroids. Initial rates of enzyme synthesis are higher with steroids as inducer than with camphor. Camphor also seems to act as inducer for the steroid enzymes, because preincubation with camphor enhances the rate of steroid metabolism by whole cells.

Degradation of L-Camphor

Apart from D(+) camphor both L and DL camphor are capable of supporting the growth of *Pseudomonas* C_1 . The extracts of L-camphor grown filtrate had a VPC pattern identical with a similar extract from D(+) camphor broth (Le Gall et al, 1963). Compounds (2) and (5) formed from L camphor had their melting points and I.R. spectra identical with the corresponding compounds from D camphor metabolism, although with an equal and opposite rotation. The FMN-coupled NADH oxidase (E_1) and lactonisation enzyme, when induced with D(+)camphor

are specific for this enantiomer and L camphor does not serve as a substrate. L-camphor-induced cells besides lactonising the L isomer, also shows some activity with the D-camphor analogues. The reason for this has been attributed to the presence of 3 per cent of D(+) camphor as an impurity in the samples of L camphor. However, the alternative possibility of induction of both the sets of enzymes cannot altogether be ruled out (Gunsalus et al 1964).

Studies with *Pseudomonas* C_3 revealed that this strain growing on glucose is inhibited by camphor and 1,2 camphyllide (7) even though this camphyllide accumulates after several hours growth in the presence of camphor. In the case of the C_1 strain this camphyllide does not accumulate when this organism is grown on camphor. Even then the cell-free extracts are capable of catalysing this reaction. During growth of the strain C_1 on camphor, both neutral and acidic compounds were found to accumulate. In the case of the strain, C_3 , growing on camphor the first product to accumulate is 1,2 camphyllide. Other intermediates including 3,4,4-trimethyl-5-carboxymethyl-cyclopentenone (5) accumulate only at a later stage. The strain, C_1 , accumulates 0.1 mole of (5) when grown on camphor. The addition of camphor prevents the utilisation of this compound until all the camphor is exhausted. The C_3 strain does not exhibit such an effect. Le Gall and Gunsalus (1963) studied the rates of growth and the molar yields of products of the above two strains using succinate, acetate, glucose, D(+)camphor and (5) as the substrates. It was observed that a succinate-grown inoculum when transferred to a glucose-camphor-ammonium salt medium adapted simultaneously to both the carbon sources. Glucose is capable of

serving as a carbon source of those mutants which are incapable of growth on camphor. Nevertheless, they accumulate intermediates in the bio-oxidation pathway of camphor when grown with it as an exogenous carbon source.

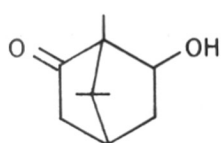
The 2,6 Diketocamphane pathway

A different pathway for degradation of camphor involving the intermediate, 2,6 diketocamphane, has been found to be present in a diphtheroid strain, T₁, isolated by enrichment on Terpin hydrate. This organism has been found to grow on terpin hydrate, α -terpineol as well as on camphor. Cells grown on acetate or fructose are simultaneously adapted to the terpenoids. Terpin hydrate-grown or induced cells oxidise α -terpineol and acetate without any lag phase (Baum & Gunsalus, 1962).

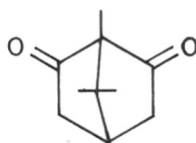
The major compound isolated from methylene chloride extract of fermented broth of this organism with camphor as substrate, was found to be a hydroxy camphor, not identical with the ones isolated in the case of *Pseudomonas* C₁. Furthermore, this hydroxy camphor and camphor are oxidised by camphor-grown diphtheroid cells which do not oxidise any of the intermediate in the *Pseudomonas* pathway (Kay et al 1962). The above compound was identified as 6 hydroxy-camphor (12). No other compound was found to accumulate in quantities sufficient for isolation and characterisation. The other probable intermediates (2,6 diketocamphane (13) and α -campholenic acid (14)) were prepared synthetically. D-Camphor-grown cells were capable of oxidising these intermediates (Chapman et al 1963). A more careful examination of the methylene chloride extracts demonstrated the presence of 2,6 diketocamphane and a mixture of

acids one of these being α -campholonic acid. Extracts of D-camphor-grown cells catalyse the oxidation of NADH in presence of 2,6 diketocamphane.

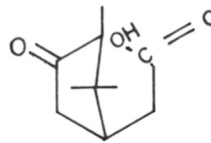
FIG. IV



6-HYDROXY CAMPHOR
(12)



2,6-DIKETO CAMPHANE
(13)



α -CAMPHOLONIC ACID
(14)

The enzyme responsible for the dehydrogenation of 6-hydroxy-camphor to 2,6 diketocamphane has been purified to physical homogeneity and crystallised (Kuo et al 1964). It requires a divalent cation (i.e., Hg^{2+}) for maximal activity. It was found to react with a wide range of substrate analogues. The enzyme was found to oxidise NADH with all the five possible camphor diketones but neither with monoketones (2 and 6), nor with any hydroxyketone. In the forward reaction of NAD reduction all exoalcohols tested were reactive but with widely differing rates. On the basis of the kinetic data a multiple enzyme site has been postulated.

Microbiological degradation of citronellol

Using enrichment culture technique Seubert (1960) isolated an organism, *Pseudomonas citronellolis*, growing on citronellol as

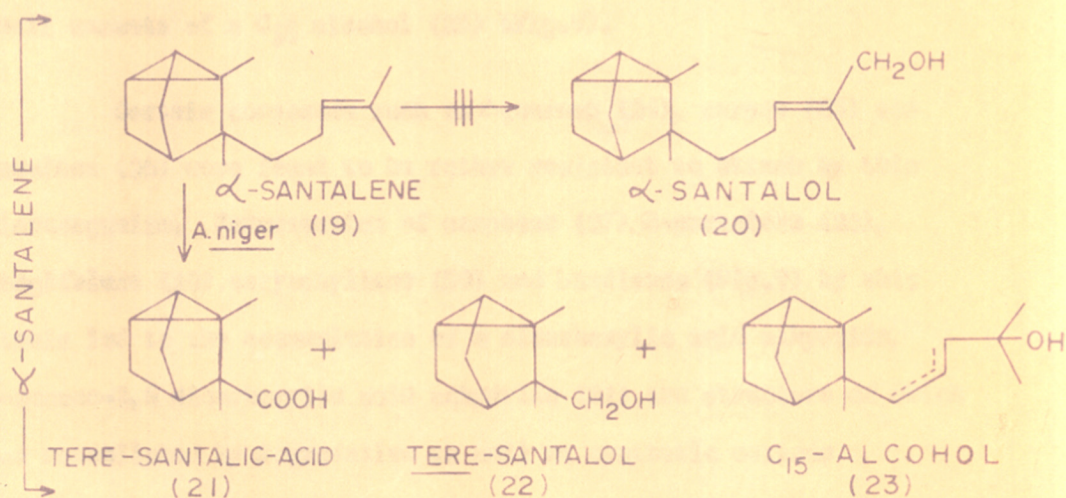
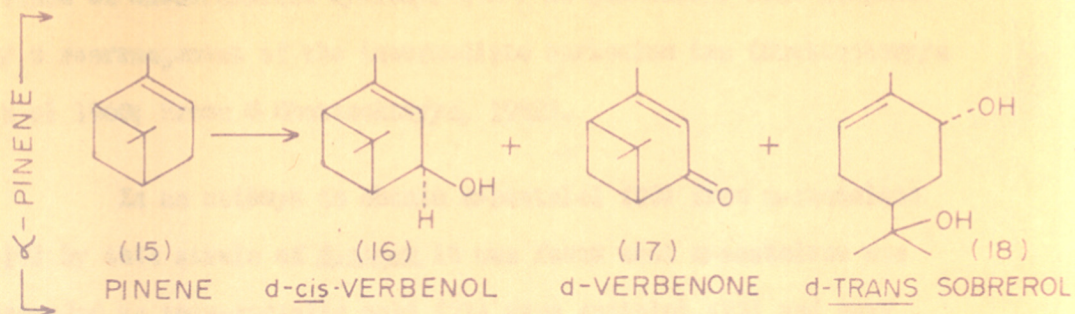
the sole carbon source. This organism oxidised citronellal and farnesol, as well as a number of other compounds which could possibly be intermediates in the degradation of this compound. Extending the studies to the metabolism of acetate in dried cells using labelled carbon, he also demonstrated that citronellal is partially converted to an ether soluble fluorescent pigment and to a number of amino acids. The nature of this fluorescent pigment is not known.

Fungal transformation of terpenoid hydrocarbons

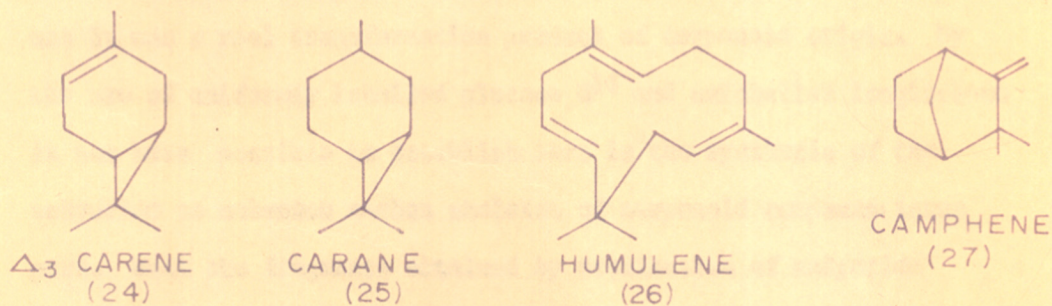
Bhattacharyya and co-workers started a systematic investigation on microbiological transformation of terpenes with a view to converting the terpenoid hydrocarbons into useful and economically important oxygenated derivatives. Their investigations were prompted by the observation of some earlier workers (Bose 1938, Sadgopal and Varma 1952) that yields of essential oil from the agarwood (Aquilaria agallocha) depend on the degree of microbial infection, the main infectants being Aspergilli, Penicillia and Fusaria. From the infected agarwood a number of odoriferous compounds (agarol, dihydroagarofuran, β -agarofuran) has been isolated by Bhattacharyya and his colleagues (Bhattacharyya and Jain, (1959), Maheshwari et al (1963)). The noninfected agarwood consists mainly of hydrocarbons.

The fermentation of α -pinene (15) by Aspergillus niger NCIM 612 yielded mainly d-cis-verbenol (16) and small amounts of d-verbenone (17) and d-trans-sobrerol (18) (Fig.V). Verbenol and verbenone were supposed to be formed by allylic oxygenation and

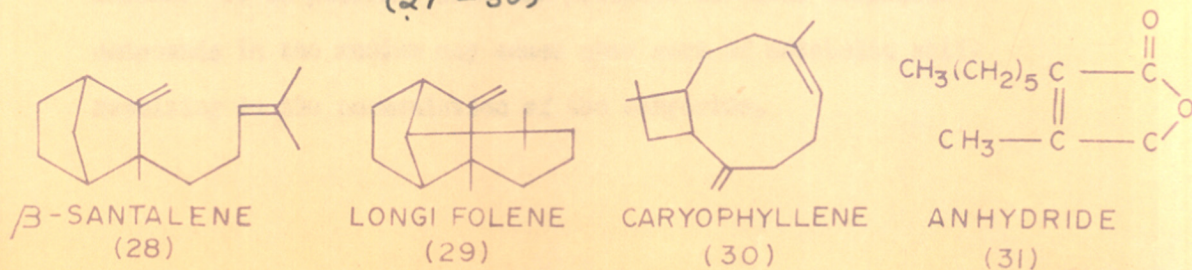
FIG. V MICROBIOLOGICAL TRANSFORMATIONS BY A. NIGER



COMPOUNDS RESISTANT TO ATTACK BY A. niger (24-26)



COMPOUNDS STIMULATING THE ACCUMULATION OF ANHYDRIDE (31) (27-30)



oxidation of resulting alcohol, while ~~trans~~-sobrerol was formed by attack of electrophilic hydroxyl (OH^+) at the double bond followed by a rearrangement of the intermediate carbonium ion (Bhattacharyya et al 1960; Prema & Bhattacharyya, 1962).

In an attempt to obtain α -santalol (20) from α -santalene (19) by this strain of *A.niger* it was found that α -santalene was degraded to ~~trans~~-santallic acid (21) ~~trans~~ santalol (22) and very small amounts of a C_{15} alcohol (23) (Fig.V).

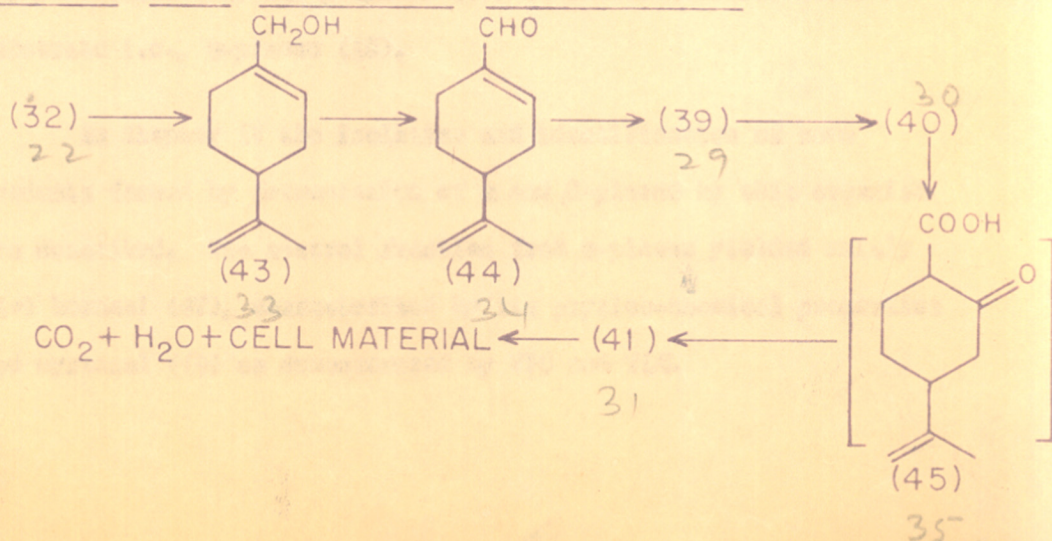
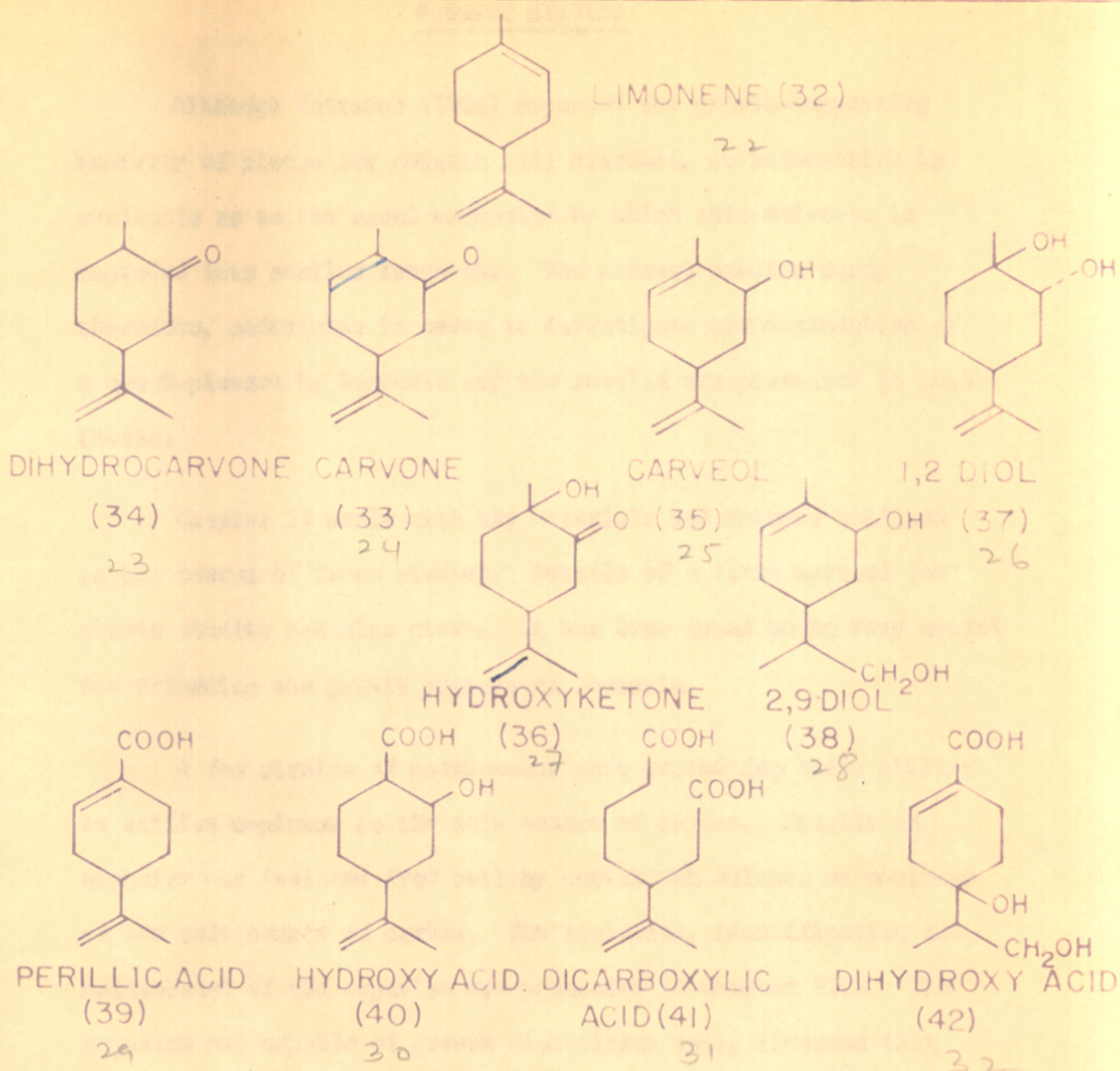
Certain compounds such as Δ_3 carene (24), carene (25) and humulene (26) were found to be rather resistant to attack by this microorganism. Fermentation of camphene (27), β -santalene (28), longifolene (29), caryophyllene (30) and δ -cadinene (Fig.V) by this strain led to the accumulation of a dicarboxylic acid anhydride, 2-nonen-2,3 dicarboxylic acid anhydride (31), the structure of which was established by degradative as well as synthetic methods (Bhattacharyya et al, 1963). As the product did not bear much resemblance to the terpene substrate, it was suspected whether or not it was a real transformation product of terpenoid origin. By the use of uniformly labelled glucose C^{14} and unlabelled longifolene it has been possible to establish that in the synthesis of the anhydride no unbroken carbon skeleton of terpenoid compound takes part. Both the fragments obtained by ozonisation of anhydride (pyruvic acid and α -ketocaprylic acid) were labelled to the same extent. It is possible that the presence of these terpenoid compounds in the medium may cause some sort of metabolic shift resulting in the accumulation of the anhydride.

Fermentation of terpene hydrocarbons by bacteria

A *Pseudomonas* isolated from soil by enrichment on α -pinene was found also to grow on limonene (32) as the sole source of carbon (Bhattacharyya et al 1964). The fermentation of limonene by this strain yielded a number of neutral and acidic compounds which were separated into their components by the use of various physico-chemical techniques. The neutral fraction yielded carvone (33), dihydrocarvone (34), carveol (35), p-menth-8-ene-1-ol-2-one (36), p-menth-8-ene-1,2-diol (37) and p-menth-6-ene-2,9-diol (38). From the acidic fraction perillic acid (39), 2-hydroxy-p-menth-8-ene-7-oic acid (40), isopropenyl pimelic acid (41) and a dihydroxy acid (42) have been isolated (Fig.VI).

Growth and adaptive enzyme studies indicated that none of the neutral compounds (33-38) lies on the direct pathway for the degradation of limonene. On the other hand perillic acid (39), hydroxy acid (40) and the dicarboxylic acid (41) were found to be oxidised by limonene-grown cells. Glucose-grown cells of this strain mainly converted limonene (32) to perillic acid (39) and small amounts of perillic alcohol (43). Based on the above observations a pathway for the degradation of limonene has been suggested (Fig.VI).

The enzymes responsible for these conversions have been demonstrated to be present in cell-free extracts. The first enzyme responsible for limonene hydroxylation has been found to be present in 100,000 g sediment and catalysed the hydroxylation of limonene in the presence of NADPH and O_2 . The 100,000 g supernatant has been found to contain the enzymes converting both perillic alcohol (43) and perillic aldehyde (44) to perillic acid (39). This fraction has been also found to catalyse the conversion of perillic acid to β -isopropenyl pimelic acid (41) in the presence of ATP, CoASH and NAD.



PRESENT STUDIES

Although Matthews (1924) reported the growth-supporting activity of pinene for certain soil microbes, no information is available as to the exact mechanism by which this molecule is degraded into smaller fragments. The present studies were, therefore, undertaken in order to investigate the degradation of α and β -pinenes by bacteria and the results are presented in this thesis.

Chapter II deals with the materials and methods utilised in the course of these studies. Details of a flask devised for growth studies are also given. It has been found to be very useful for recording the growth pattern of bacteria.

A few strains of pseudomonas were tested for their ability to utilise α -pinene as the sole source of carbon. Finally an organism was isolated from soil by enrichment culture on α -pinene as the sole source of carbon. The isolation, identification and maintenance of the organism are presented in Chapter III. The organism was capable of growth on β -pinene (46), limonene (32), Δ^1 -p-menthene (74) and p-cymene (97) in addition to its usual substrate i.e., α -pinene (15).

In Chapter IV the isolation and identification of some products formed by fermentation of α and β -pinene by this organism are described. The neutral fraction from α -pinene yielded mainly d(+) borneol (47), characterised by its physico-chemical properties and myrtenol (49) as demonstrated by VPC and TLC.

The acidic fraction gave a dicarboxylic acid (53) isolated from the total acid mixture through its insoluble dicyclohexylamine salt. The mother liquors after separation of this acid has been further fractionated by conversion to methyl ester and partitioning between petroleum ether and 90% aq. methanol. The nonpolar fraction has not been found amenable to usual methods of separation, but the presence of myrtenic (58), phellandric (59) and perillic acids (39) in this fraction has been demonstrated by comparative vapour phase chromatography of methyl esters. In addition a fatty acid comprising 50% of this nonpolar fraction has been isolated.

The polar methyl esters have been found to contain oleuropeic acid methyl ester (51) and another hydroxy acid methyl ester (60) which have been separated and identified on the basis of their spectral data and simple chemical reactions. In addition, another dihydroxy acid (42) has been isolated in very small amounts.

The fermentation of β -pinene gave (-) borneol, the saturated dicarboxylic acid (53) and appreciable amounts of (-) oleuropeic acid (50). Another hydroxy acid methyl ester with the tentative structure (60) has also been isolated in small amounts. In addition a fatty acid and a mixture of monocarboxylic acids similar to that from α -pinene have also been obtained.

Adaptive enzyme studies presented in Chapter V will indicate that borneol, myrtenol or even oleuropeic acid are not oxidised by pinene-grown cells, whereas the monocarboxylic acid fraction is oxidised at a fast rate. The dicarboxylic acid (53) was found to be oxidised at a very slow rate.



Cross adaptation studies also presented in this chapter would indicate that cells when grown on α or β -pinene are also adapted to limonene and Δ -1-p-menthene. The cells grown on limonene oxidise Δ -1-p-menthene at comparative rates, but α - and β -pinene are oxidised only slowly. Based on the manometric studies using limonene (32), Δ -1-p-menthene (74), perillyl alcohol (43) perillic acid (39), phellandrol (75), phellandric acid (59), β -isopropenyl pimelic acid (41), β -isopropyl pimelic acid (53) as substrates and α and β -pinene grown cells, probable pathway(s) for the degradation of pinenes have been arrived at. The presence of an alcohol dehydrogenase catalysing the dehydrogenation of perillic alcohol (43), phellandrol (75) and eleuropeic alcohol (80) has been presented. An aldehyde dehydrogenase catalysing the conversion of the corresponding aldehydes to acids has also been found to be present in these cells.

In Chapter VIa the growth of this organism on a number of model substrates is presented. These compounds included terpenoid hydrocarbons, their oxygenated derivatives and other simple compounds some of which are probable intermediates in the degradation of pinenes. These studies, on one hand, lend support to the concepts about the degradative pathways developed in Chapter V and, on the other, provide information regarding the structural features and functional groups essential in a molecule before it can serve as a sole source of carbon for this organism.

In Chapter VIb these studies regarding the essential structural requirements have been extended to aromatic model compounds related to p-cymene (97). For serving as the growth

substrate the simplest compound has been found to be 1-methyl-4-ethyl benzene (102). It is possible to alter the side chain in position 4 to a limited extent without affecting the growth supporting activity. However the alternation of the methyl group in position -1 and further substitutions on the benzene ring are not tolerated. An introduction of an oxygen function in position 8 of the p-cymene molecule or p-menthenoid compounds completely abolishes the growth supporting activity.

Chapter VII summarises the results obtained during the course of these studies.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS & METHODS

All the chemicals used for the preparation of media were of chemically pure grade and those used for enzymic purposes were of analytical reagent grade. α -Pinene was a B.D.H. product which was further purified by a batch-stripping fractional distillation at atmospheric pressure through a 2" x 4' column. The fraction boiling between 153.5°-154.5°C/710 mm was collected and further purified by refluxing over metallic sodium and distillation. The α -pinene used in these studies was 99.5 per cent pure according to the VPC data and had a rotation of (α_D +21°). Since it is not possible to get optically pure d α -pinene, this material was used for all the studies in this series. Two samples of β -pinene were used in these studies of which one was supplied by Dr. S. C. Bhattacharyya, Essential Oils Division of this Laboratory, and had a b.p. 162.5°-164°C/710 mm, (α_D -17.6°). The other sample of β -pinene was a technical grade material from Fluka and was found to be 90 per cent pure by VPC. It was further purified by fractionation through an efficient spinning band column. A reflux ratio of 1:25 was maintained throughout. The fraction boiling at 162°-163.5°C/711 mm was collected and found to be 99.5 per cent pure by VPC, (α_D -18.6°). L- α -Pinene was prepared from this β -pinene by refluxing over gum resin for 24 hours and fractionation (Austerweil, 1926). The pure l- α -pinene had a b.p. 153°-154°C/711 mm, (α_D -39.6°). The limonene used in these studies was prepared by fractionation of orange oil, the fractions b.p. 172°-174°C/710 mm being used. The other

sample of limonene used was a Fluka product (99% pure - VPC).
The p-cymene was obtained from M/s. Riedel de Haan Ltd., Germany.

A sample of oleuropeic acid was kindly supplied by Dr. Y. Mazur of Weizmann Institute of Science, Israel. The samples of Δ^1 -p-menthene, (+) phellandrol and (+) phellandral were obtained through the kind courtesy of Dr. Porsch of Drageco, W. Germany. The sample of phellandric acid was prepared by silver oxide oxidation of (+) phellandral. The samples of Δ^8 -p-menthene, Δ^1 -7-p-menthene and Δ^1 (7),8-p-menthadiene were obtained from Dr. E. Klein of Drageco Laboratories.

Maintenance and propagation of culture:

The organism growing on α -pinene as the sole source of carbon was propagated on a nutrient agar slant of the following composition (Mackie and Mc Cartney, 1949).

Peptone (Armour)	1 gm
NaCl (B.D.H.).....	0.5 gm
Yeast extract (Oxoid).....	0.5 gm
Beef extract (Difco).....	0.5 gm
Agar	2 per cent
Total volume.....	100 ml

The first four constituents were dissolved in water and the pH was adjusted to 7.0 with 4N sodium hydroxide. The final volume was made up to 100 ml. Two per cent of agar (Alcaden or Difco) was added and the mixture was steamed for one hour to melt the agar. For preparation of agar slants 6-7 ml aliquots were distributed

into 19 x 150 mm Pyrex test tubes and autoclaved at 15 p.s.i. (120°C for 20 min.). The tubes were then slanted while hot and allowed to solidify and remain undisturbed overnight. The slants were again steamed for one hour to destroy any germinated spores. The tubes were reslanted and allowed to solidify. These slants were inoculated from broth or stock culture and incubated at 28°C for 24 to 48 hours.

For purification of the culture by streaking, nutrient agar of the above composition was poured aseptically while hot in previously autoclaved (20 lb for one hour and then at dry heat 160°C for one hour) petri dishes. Under certain conditions the culture can be propagated even on glucose-mineral salt-agar slants. The composition of mineral salts is described below and to this were added 1% glucose and 2% agar and the slants were prepared in the usual way.

Mineral salt medium for growth and fermentation

The organism was grown in a chemically defined medium described by Seubert (1960) having the following composition.

1) Dipotassium hydrogen phosphate (Danpha grade, India)	63 gm
2) Potassium dihydrogen phosphate (B.D.H.).....	18.2 gm
3) Ammonium nitrate (E. Merck)	10 gm
4) Magnesium sulphate (B.D.H.) anhydrous	1 gm
5) Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ - E. Merck)	1 gm
6) Ferrous sulphate heptahydrate (B.D.H.)	1 gm
7) Manganese sulphate (Riedel de Haan)	6 mg
8) Sodium molybdate (B.D.H.)	6 mg

Made up to 10 litres, pH 7.0.

All the constituents were dissolved separately, excepting for the phosphates which were dissolved together and diluted to 8 litres. Aliquots from the solution of other mineral salts were mixed together and then added to phosphate solution and the total volume made up to 10 litres. Aliquots of 100 ml medium were added to 500 ml Erlenmeyer flasks which were plugged and autoclaved (15 p.s.i. for 20 min). A 2-5% inoculum was added to each flask and 0.3-0.5 ml terpene substrates were added as carbon source. The flasks were incubated at $28^{\circ} \pm 1^{\circ}\text{C}$ on a rotary shaker at 220 r.p.m. Fresh aliquots (0.3-0.5 ml) of hydrocarbon substrates were added every 24 hr to compensate for the loss due to evaporation.

Extraction and separation of components

At the end of the incubation period (24 - 72 hr), the flasks were pooled together and extracted once with ether. The broth was then acidified with 6N HCl and reextracted with ether thrice. All the ether layers were combined and freed of suspended cells (which came in this layer after acidification and extraction) by shaking with hyflo supercel. The supercel cake was washed again with ether and these washings were combined with the main ether layer.

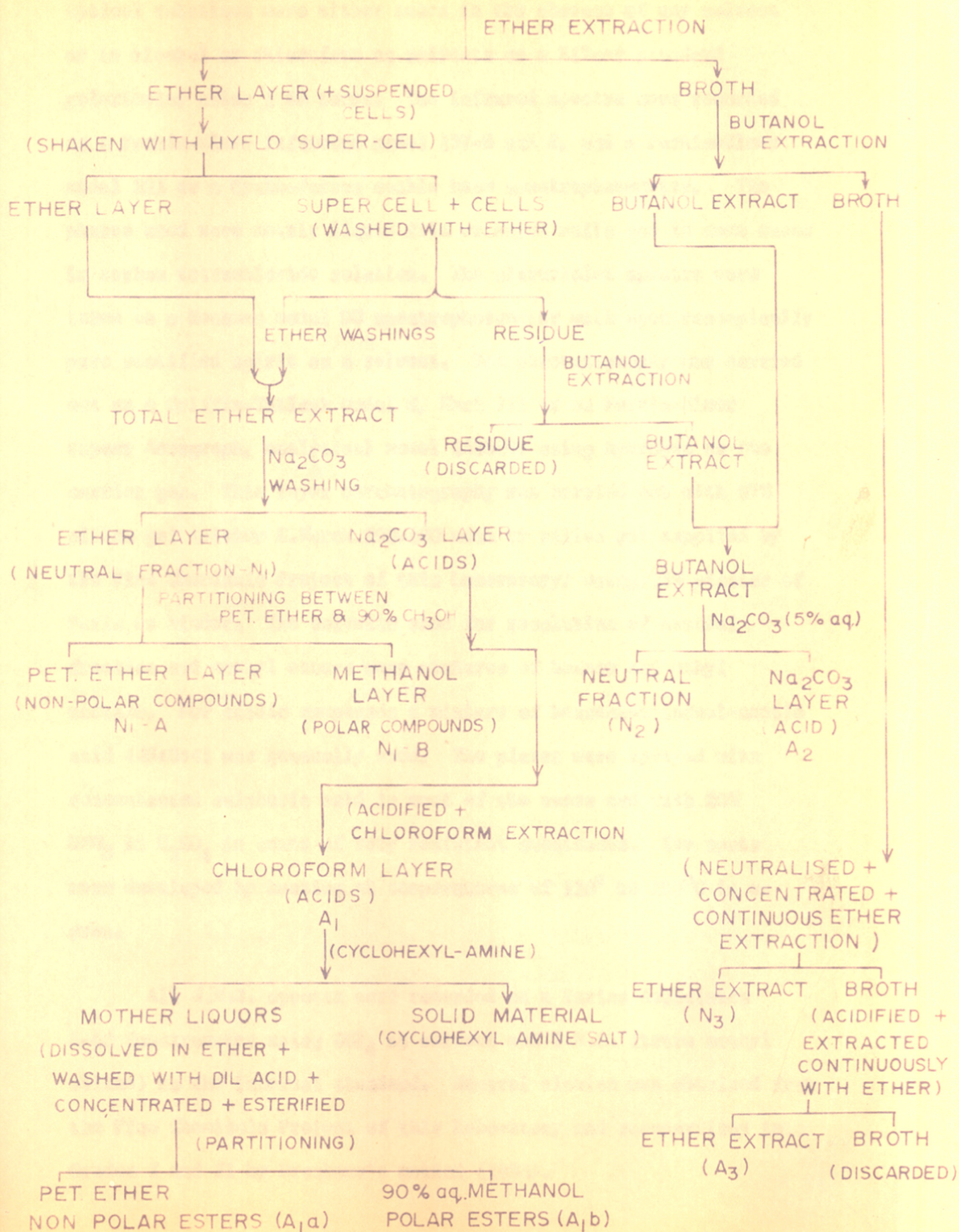
The clear yellow broth and the supercel cake containing the cells were extracted with butanol. The aqueous phase was neutralised first with 10% sodium hydroxide solution and then with ammonia and concentrated to a small volume. The concentrate was extracted continuously with ether to give highly polar neutral compounds (N_3). The aqueous phase was acidified and again extracted continuously with ether to give highly polar acidic fractions (A_3).

The ether and butanol extracts were separated into neutral (N_1 and N_2) and acidic fractions (A_1 and A_2) by washing with aqueous 5% sodium carbonate. The acids were regenerated from the sodium carbonate layer by acidification and taken up in ether or chloroform. The neutral components were further subjected to a four-transfer counter current partition between petroleum ether and 90% methanol (Prerna & Bhattacharyya, 1962a). These fractions were then purified by chromatography, fractional distillation and sublimation as described in Chapter IM. The purity of the fractions were established both by TLC and VPC. —

The acids forming insoluble cyclohexylamine salts were separated by treatment of the total acids fraction with cyclohexylamine in acetone. The supernatant after filtration of the insoluble salts was taken up in ether and freed of cyclohexylamine by washing with dilute hydrochloric acid. The ether layer was dried (sodium sulphate) and concentrated. The concentrate was converted to methyl esters by treatment with diazomethane in ether. The polar (A_1 -b) and non-polar (A_1 -a) methyl esters were separated by a partition between petroleum ether and 90% aqueous methanol as described earlier. The methyl esters from these two layers were further separated by chromatography over alumina and/or fractional distillation. The separation and identification of these compounds were monitored by various physico-chemical methods such as TLC, VPC and various other spectroscopic methods detailed in a later chapter. The extraction procedure and the methods of separation are summarised in Fig.VII.

Fig VII

EXTRACTION PROCEDURE FERMENTED BROTH AND CELLS



All the melting and boiling points reported are uncorrected. Optical rotations were either taken in the absence of any solvent or in alcohol or chloroform as solvents on a Hilger standard polarimeter using 1 dm tubes. The infrared spectra were recorded on a Perkin-Elmer infracord model 137-B and E, and a Perkin-Elmer model 221 or a Grubb-Parson double beam spectrophotometer. The phases used were mostly liquid film or Nujol mulls and in some cases in carbon tetrachloride solution. The ultraviolet spectra were taken on a Beckman model DU spectrophotometer with spectroscopically pure rectified spirit as a solvent. Gas chromatography was carried out on a Griffin-Tatlock model G, Mark III or on Perkin-Elmer Vapour Aerograph, analytical model (A350B) using hydrogen as the carrier gas. Thin layer chromatography was carried out with 85% silica gel (either E. Merck 200-400 mesh or silica gel supplied by the Fine Chemicals Project of this Laboratory) using 15% plaster of Paris as binder. The solvents used for resolution of neutral fraction and methyl esters were mixtures of hexane and ethyl acetate. For acidic compounds a mixture of benzene-methanol-acetic acid (45:8:4) was generally used. The plates were sprayed with concentrated sulphuric acid in most of the cases and with 20% HNO_3 in H_2SO_4 in cases of very resistant substances. The spots were developed by heating at temperatures of 110° to 160°C in an oven.

All N.M.R. spectra were recorded on a Varian Associates A-60 Spectrometer using CCl_4 as solvent and T.M.S. (tetra methyl silane) as the internal standard. Neutral alumina was obtained from the Fine Chemicals Project of this Laboratory and standardised into Grades I and II by Brockman's method (1941).

Growth Studies

Growth studies were recorded either visually or with a Klett-Summerson colorimeter, using a flask described below, which was developed in the course of these studies. This flask has been found specially suitable for work with insoluble substrates.

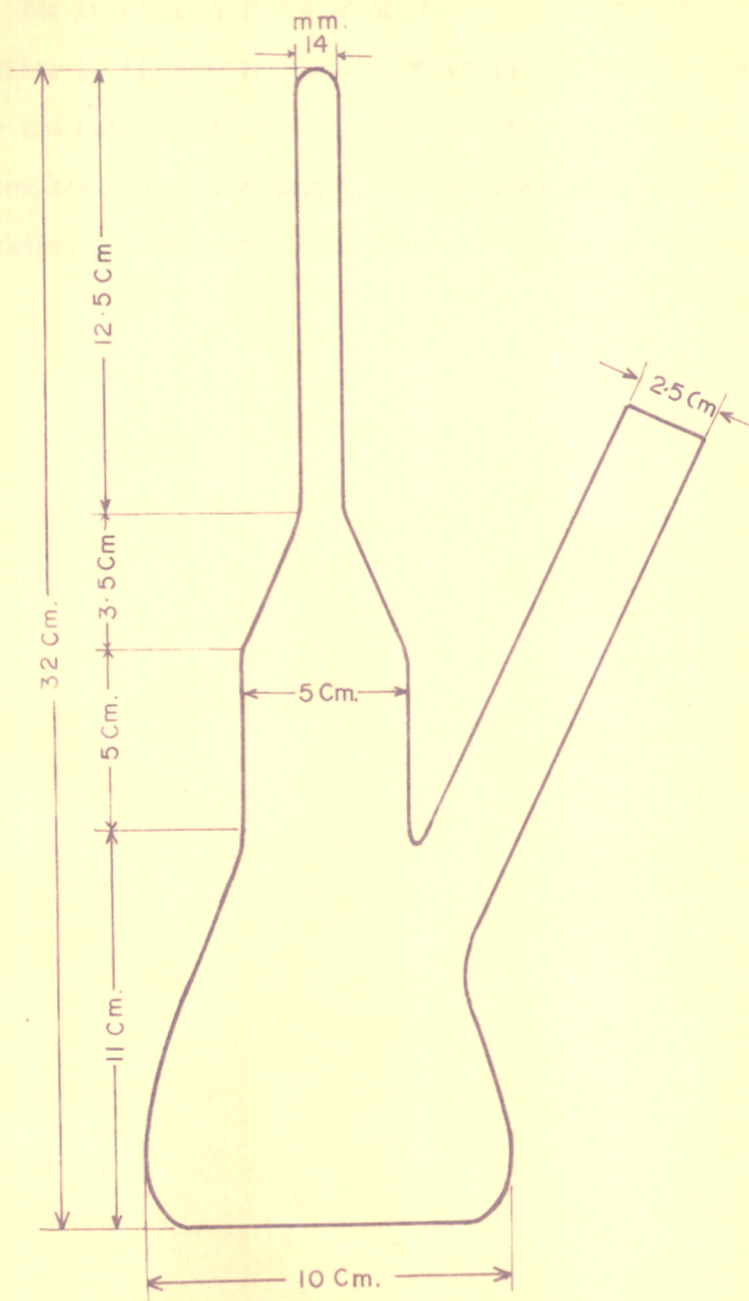
The flask (Fig.VIII) can be conveniently prepared from Erlenmeyer flasks of 500 ml capacity. The top of the flask is drawn out and optically matched tubes (14 x 120 mm) are fused as shown in diagram. The side neck is made by fusing a 25 x 120 mm boiling tube at an angle of 60° to the horizontal. The capacity of the flask from the tips of the optically matched tubes to the point of junction of side neck is adjusted to 110 ml by blowing a bulb in between. The flasks are filled with medium, sterilised, inoculated through the side neck and incubated at 28°C on a rotary shaker. At suitable intervals the flasks are removed and readings recorded by inserting the tube directly into the colorimeter by inverting the flasks. This method of recording eliminates any chance of contamination and simplifies the cumbersome process of withdrawing aliquots for turbidity measurements.

Manometric studies

Measurements of O₂ uptake by cell preparations were carried out by manometric techniques using Warburg flasks (25 ml capacity) with a single side arm.

The flasks in a total volume of 3 ml contained 0.2 ml 20% KOH in central well, 1.5 ml cell suspension containing 1-2 mg

FIG. VIII



dry wt. cells, M/20 phosphate buffer, 1 ml and water to make the volume 3 ml. The substrates (5 to 20 umoles) and other substances were added either by tipping from side arm or incorporating them directly into the main flask (in each case the volume was kept at 3 ml). The temperature of the bath was maintained at 28°C and flasks were shaken in this bath (110 strokes/min with 2 cm strokes).

CHAPTER III

ISOLATION, IDENTIFICATION, MAINTENANCE AND GROWTH BEHAVIOUR OF THE ORGANISM

CHAPTER III
ISOLATION, IDENTIFICATION, MAINTENANCE & GROWTH BEHAVIOUR
OF THE ORGANISM

a) Screening of some Pseudomonas strains for their ability to grow on α -pinene as the sole carbon source:

The following organisms were obtained from the National Collection of Industrial Microorganisms of this Laboratory. The cultures were maintained on nutrient agar slants of the composition described earlier.

- 1) Pseudomonas fluorescens
- 2) P. aeruginosa
- 3) P. fluorescens (A.312)
- 4) P. fluorescens (6-BC)
- 5) Pseudomonas (6751)
- 6) Pseudomonas (Trans MRR)
- 7) Pseudomonas (TCL, MRR)
- 8) Pseudomonas (BGV isolate)

After 24 hr growth on nutrient agar two loopfuls of each organism were inoculated into two flasks containing mineral medium of the composition described earlier. To one of the two flasks 0.2 ml of α -pinene was added and the other was treated as control. Both the flasks were incubated on a rotary shaker at a temperature of 28°C. At the end of every 24 hours, small amounts of α -pinene (0.1-0.2 ml) were added to replenish the stock. Visual observation of the flasks was made for about 10 days. No growth was observed

in any of the flasks during the experimental period indicating the inability of the above organisms to utilise α -pinene as sole source of carbon for their growth.

b) Isolation of bacteria capable of growth on α -pinene as the sole source of carbon by enrichment culture technique:

Garden soil (5 g) was suspended in 25 ml of distilled water and homogenised on a shaker for 2 to 3 hours. At the end of this period the bigger particles of soil were removed by filtration through a cheese cloth. The resulting filtrate was centrifuged to sediment the smaller particles of soil and bacteria. The sediment was washed once more with the mineral salt medium described earlier to remove any nutrient material from soil remaining in it. Finally the sediment was suspended in about 25 ml of the same medium. About 3 to 4 ml of the suspension were inoculated into 100 ml of mineral salt medium. The flasks were incubated on a shaker at $29^{\circ} \pm 2^{\circ}\text{C}$ after addition of 0.2 ml of α -pinene. At the end of five or six days 5 to 10 ml aliquots from this flask were transferred into another flask containing α -pinene and incubated as before. At the end of three such transfers heavy growth was observed in the flasks. However, the transfers were continued for two to three more times. Microscopic examination of the contents revealed the presence of multitude of organisms, mostly small rods.

c) Ability of the organism to grow on α -pinene from vapour phase

As it is difficult to prepare pinene agar slants due to the highly insoluble and volatile nature of α -pinene, it was decided to test the ability of the organism to utilise α -pinene from vapour phase. Petri dishes containing mineral salt-agar medium were

prepared in the usual manner by incorporating 2% agar in the mineral salt medium. A loopful of inoculum from liquid culture was spread on to the agar surface and plates were incubated at 28°C in a desiccator containing α -pinene. At the end of 48-72 hr, growth was apparent on the surface indicating that the isolates utilize α -pinene from the vapour phase. Similarly inoculated plates incubated in the absence of pinene failed to exhibit any growth.

d) Purification of the culture:

It was observed that the organism retains its capability to grow back on pinene even when maintained on a nutrient agar surface for a few generations. Further purification of the culture was accomplished by streaking on nutrient agar surface. The colonies were picked up from the nutrient agar surface and propagated on nutrient agar slants only. The growth from nutrient agar slants was retransferred on to α -pinene-salt medium and the colonies capable of growth on α -pinene were restreaked. This operation was repeated several times before the experimental strain was obtained in a homogeneous form.

e) Identification of the organism:

The organism metabolising α -pinene was identified as belonging to the genus "*Pseudomonas*" as per Bergey's Manual of Determinative Bacteriology, VIIIth Edition (Breed et al, 1957). The various morphological, cultural and biochemical characteristics of the organism are summarised in Table 1.

TABLE 1
IDENTIFICATION OF THE ORGANISM

Sr. No.	Test	Observation
A. Morphological & Cultural		
1	<u>Nutrient broth</u>	
	Surface	ring
	Clouding	moderate
	Odour	foul
	Sediment	compact
	Abundant of sediment	scanty
2	<u>Agar colonies</u>	
	Form	circular
	Surface	smooth
	Edge	entire
	Elevation	raised
	Optical character	translucent
3	<u>Agar stroke</u>	
	Growth	abundant
	Form	filiform
	Lustre	glistening
	Chromogenesis	no fluorescence
	Odour	foul
	Consistency	butyrous
	Medium	unchanged
4	<u>Gelatin liquefaction</u>	negative

(contd.)

TABLE 1 (contd.)

Sr. No.	Test	Observation
5	<u>Vegetative cells</u>	
	Form and arrangement	rods, thin
	Diameter	less than 0.5 μ
	Size	0.5 μ x 1.5 μ
	Gram stain	negative
	Flagella	present
	Motility	both in broth and agar slants at 30°C and 37°C
	Capsules	absent
	Chains	absent
	Sporangia	absent
	Endospores	absent
	Optimum growth	37°C
B.	<u>Physiological</u>	
1	Biological relationship	not autotrophic
2	Relation to free oxygen	aerobic to microaerophilic, not anaerobic
3	In nitrate media	nitrite, but no gas
4	Chromogenesis	none
5	Other photic characters	none
6	Indole	negative
7	Hydrogen sulfide	negative

(contd.)

TABLE 1 (contd.)

Sr. No.	Test	Observation
C	<u>Additional tests</u>	
1	Methyl red	negative
2	V.P. Test	positive
3	Citrate utilization	positive
4	Milk peptonization	positive
5	Optimum pH for growth	6.8-7.2 and does not grow below 6 and above 8
6	NaCl tolerance	Does not tolerate more than 2% salt concentration
7	Peptone water + Glucose	enhanced growth
8	Yeast water + 10% ethyl alcohol	no growth
9	Glucose fermentation with peptone water under microaerophilic condition	slight growth, no acid, no gas, becomes alkaline
10	Fermentation of glucose under aerobic condition	Growth and acid, no gas
11	Fermentation of lactose under aerobic condition	Growth, no acid, no gas, not alkaline
12	Gas in peptone water	no
13	Acidity from fructose	positive
14	NH ₃	negative
15	Starch hydrolysis	positive
16	Catalase	positive
17	Utilization of phenol	negative
18	Agar digestion	negative

f) Maintenance of the culture

It is possible to maintain the culture for a few generations on the surface of nutrient agar by serial transfers at 15 to 20 days intervals. However, beyond five or six such transfers the organism, though viable on nutrient agar, loses its capacity to grow back on α -pinene. It is possible to maintain the organism for very long periods on the surface of nutrient agar in case the surface growth is covered with a layer of sterilised liquid paraffin. In this fashion it has been possible to keep this organism viable for very long periods (ranging between 9 to 20 months in some cases). In most of the cases the organism was found to retain its capacity to grow back on α -pinene. Incubation temperature of paraffin covered slants was found to be an important factor. The slants stored at room temperature (25° - 30°C) were found to retain their viability better than the slants stored at 0° , 15° or 24°C . The strain showed better response to an incubation temperature of 24° and deteriorated rapidly at 15° and 0°C , the last incubation temperature giving the least survival.

For further work the cultures were preserved under paraffin and stored at room temperature. The nature of the constituents of nutrient agar slants seems also to be important for the survival rate. The slants prepared from Armour peptone were found to give better results than other varieties.

For routine work the culture was maintained by regular transfers in liquid culture. However, considerable amount of variations in the behaviour of this organism to growth on substrates other than α -pinene was observed in these shake cultures.

Growth behaviour of the organism

The organism has been found to grow on a number of carbohydrates and other compounds freely. These include glucose, sucrose, citrate, succinate, malate and other members of the tricarboxylic acid cycle. The growth response on isobutyric acid, propionic acid, and acetate has also been positive but requiring long periods for adaptation.

In addition to these compounds, the organism utilises a number of terpenoid hydrocarbons as growth substrates. These are α -pinene, β -pinene, limonene, p-cymene and Δ 1-p-menthene. Though the growth behaviour of the organism will be discussed in detail in a later chapter, it is worthwhile to mention a few characteristics here.

The organism when grown on α -pinene and β -pinene and transferred from these substrates to limonene, also grew on the latter substrate freely. But cells grown on limonene for a few generations refused to grow on pinenes without a prolonged lag phase. In a typical experiment the cells were adapted to each of these substrates by subculturing for a few generations. At the end of these periods the cells were freed of the adhering substrates by centrifugation, washed and resuspended in sterile medium. The centrifugations and washings were done under aseptic conditions. Suitable aliquots (2 ml of a cell suspension with a turbidity of 250-260 Klett readings, Red Filter - 660 m μ) from each were inoculated in flasks containing various substrates (0.2 to 0.3 ml) and incubated in usual manner. The readings were recorded at 24 hr intervals (Table 2).

TABLE 2
Klett Readings at 660 mμ

Inoculum	Time hr	α -pinene	β -pinene	limonene
	0	34	34	34
α -pinene-grown cells	24	302, 302	315, 312	335, 350
	48	435, 435	450, 445	455, 455
	0	36	36	36
β -pinene-grown cells	24	272, 262	312, 284	342, 340
	48	405, 415	460, 450	450, 450
	0	37	37	37
limonene-grown cells	24	40, 61	55, 45	370, 360
	48	282, 340	390, 390	465, 416
Medium blank		30	30	30

CHAPTER IV

FERMENTATION OF α AND β PINENE

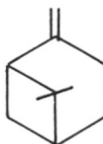
DISCUSSION

FERMENTATION OF α -PINENEOptimum conditions for fermentation

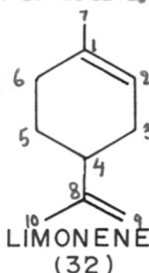
In an earlier chapter the isolation and growth requirements of a Pseudomonas metabolising α -pinene (15), β -pinene (46) and limonene (32) were presented. This organism grew remarkably well on



α -PINENE
(15)



β -PINENE
(46)



LIMONENE
(32)

these insoluble hydrocarbon substrates in a mineral salt medium at rates comparable to those on glucose. It was necessary to establish the optimum conditions for fermentations before undertaking large scale experiments to obtain the fermentation products.

In these experiments a problem arises due to the considerable volatility of the mono-terpenoid hydrocarbon substrates particularly at higher incubation temperatures. Therefore, even though the bacterium showed a temperature optimum around 37° , a sub-optimal temperature of 28° was chosen rather arbitrarily to keep the rates of evaporation as low as possible at the same time maintaining a fair growth rate.

The organism exhibited a pH optimum of 6.8-7.2. However for the fermentation a neutral pH of 7.0 was chosen to minimise the probability of acid and base catalysed non-enzymatic side reactions.

Unlike limonene, which appeared to be non-toxic to the bacterium, both the pinenes were found to be toxic especially beyond 1% levels (Table 3). It was, therefore, necessary to work with 0.3 - 0.5 per cent levels of the pinenes, replenishing these levels every 24 hr with fresh additions of 0.3 - 0.5% substrates, until a total amount of 1% was reached. It was noteworthy in this connection that with an initial level of 1% pinene, the pattern of accumulating transformation products showed some quantitative differences, the proportions of non-polar acidic transformation products being higher and the amount of polar neutral and polar acidic fractions being usually lower than that obtained by periodic replenishment of the substrate.

The amount of transformation products, both neutral and acidic, showed an increase with incubation period (Table 4). However, with prolonged incubation beyond 72 hr, considerable lyses of the cells were observed. As it will be evident later, that this may be due to the toxic effect of one of the accumulating products, borneol (47).

Isolation and characterisation of metabolites

The extraction procedure after the fermentation and the separation of metabolites into polar and nonpolar neutral and acidic products have been outlined earlier in the chapter on Materials and Methods (Fig.VII). Briefly, the fermented broth containing cells was acidified and extracted with ether and butanol. The aqueous layer was neutralised, evaporated and extracted continuously with ether again before and after acidification to recover the highly polar neutral and acidic products. Alkali washing of the earlier

ether and butanol extracts separated the acidic constituents from the neutral ones in the solvent layers. In this way six major fractions were obtained.

- a) Less polar neutral (N_1) and acidic fractions (A_1)
both from the first ether extract;
- b) More polar neutral (N_2) and acidic fractions (A_2)
from the butanol extract;
- c) Highly polar neutral (N_3) and acidic compounds (A_3)
from the continuous ether extract.

Examination of these fractions revealed the existence of at least six to eight components in each of them with a considerable amount of overlapping in the case of some constituents. Each of these fractions was further separated by solvent partitioning and/or chromatographic methods.

N_1 (Less polar neutral) Fraction:

The N_1 fraction from the first few batches was directly chromatographed over Grade I alumina. In the later experiment it was however subjected to partitioning between petroleum ether and 90% methanol to obtain two gross fractions N_1 -A and N_1 -B of lower and higher polarity. These were subjected to chromatography separately.

The following gross fractions were obtained by eluting the column with solvent systems of increasing polarity:

- N_1 -a - petroleum ether fraction
- N_1 -b - 0-25% ether-petroleum ether fraction
- N_1 -c - 50%-75% ether-petroleum ether fraction
- N_1 -d - 100% ether fraction
- N_1 -e - methanol fraction

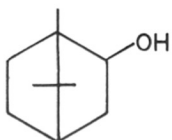
The least polar fraction, N_1 -a was found to contain unreacted α -pinene and fatty components. The recovered α -pinene had the same rotation $[\alpha]_D + 21^\circ$ as that of the starting material indicating that both the d and l isomers are oxidised by the bacterium at the same rates. There had been no enrichment of any of the isomers at the end of the fermentation.

The fatty material was separated from the volatile α -pinene fraction by fractional distillation. However, a careful rechromatography of the N_1 -a fraction on Grade-I alumina resulted in the separation of a small amount of a volatile oxygenated compound from the tail fraction which moved slightly slower than α -pinene with petroleum ether. This material analysed for one atom of oxygen only ($C_{10}H_{16}O$). The IR spectrum indicated the absence of any hydroxyl or a carbonyl function but the presence of an ether linkage ($\nu_{\max}$ 1037 cm^{-1}) and possibly a double bond ($\nu_{\max}$ 817 cm^{-1}). Since this compound was available in small amounts it was not possible to identify it.

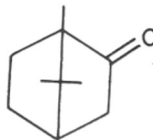
When partitioning of the N_1 (less polar neutral) fraction was resorted to, the N_1 -A fraction corresponded to N_1 -a fraction with slight contamination from N_1 -B. The N_1 -B fraction corresponded to a mixture of N_1 -b to N_1 -e as revealed by TLC.

The next fraction (N_1 -b) was found to be a mixture of at least two components with very close mobilities in TLC and could not be resolved further by rechromatography. The mixture analysed for $C_{10}H_{16}O$, showed carbonyl bands in the IR spectrum ($\nu_{\max}$ 1750, 1720 and 1695 cm^{-1}). The amount of this fraction was small and therefore not examined further.

The major neutral component was obtained from the N_1 -c fraction in a crystalline form and purified by sublimation; (m.p. 203-205°). This compound analysed for $C_{10}H_{18}O$ and was dextro-rotatory $[\alpha]_D^{+37^\circ}$. The IR spectrum (Fig. IX) indicated a hydroxyl absorption ($\nu_{\max}$ 3333, 1055 cm^{-1}), a gem dimethyl group ($\nu_{\max}$ 1377, 1355 cm^{-1}), and the absence of any double bond, a fact which was corroborated by the lack of any UV absorption. The NMR spectrum (Fig. X) indicated the presence of three methyl groups on saturated carbons (a close pair of two bands totalling 9-proton intensity between 9.17-9.22 τ , a diffuse doublet of one proton intensity 5.95-6.25 τ and methylene proton signals between 7.8-9 τ). From the above data the compound was identified as d(+)-borneol (47). The IR spectrum was superimposable on that of an authentic sample of d-borneol (Fig. IX) and there was no depression in the mixed melting points of the samples. The identity of compound (47) with d-borneol



BORNEOL
(47)



CAMPHOR
(48)

was conclusively established through the preparation of the acid phthalate m.p. 161°-162° (reported 164.5°-165°, Beilstein-9, 800) and by chromic acid oxidation to yield d(+)-camphor (48) m.p. 176°-177° (lit. 178.6°). The identity of (48) was again established by spectral comparisons and mixed melting point determination with an authentic sample.

The next fraction (N_1 -d) was a moderately volatile liquid and was distilled under vacuum at 125°-130°/4 mm (bath temp.). However,

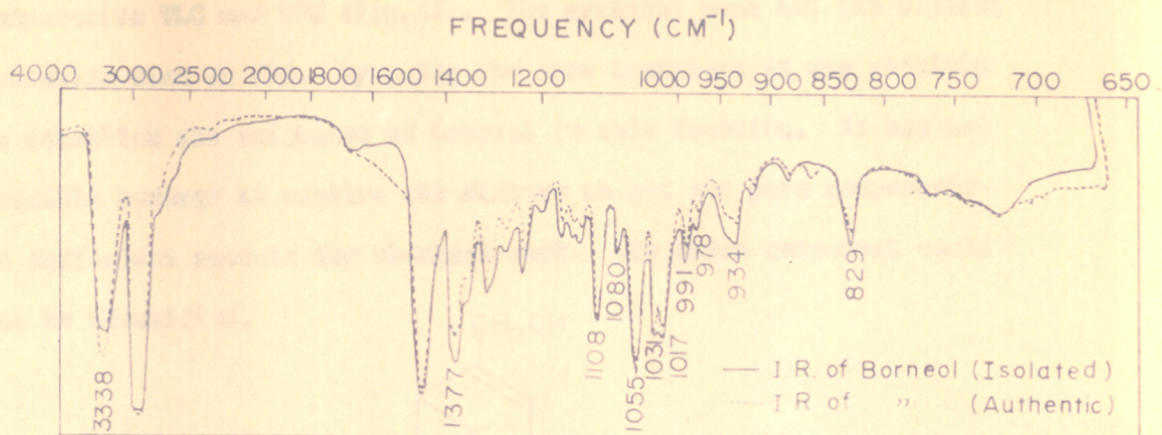


FIG. - IX

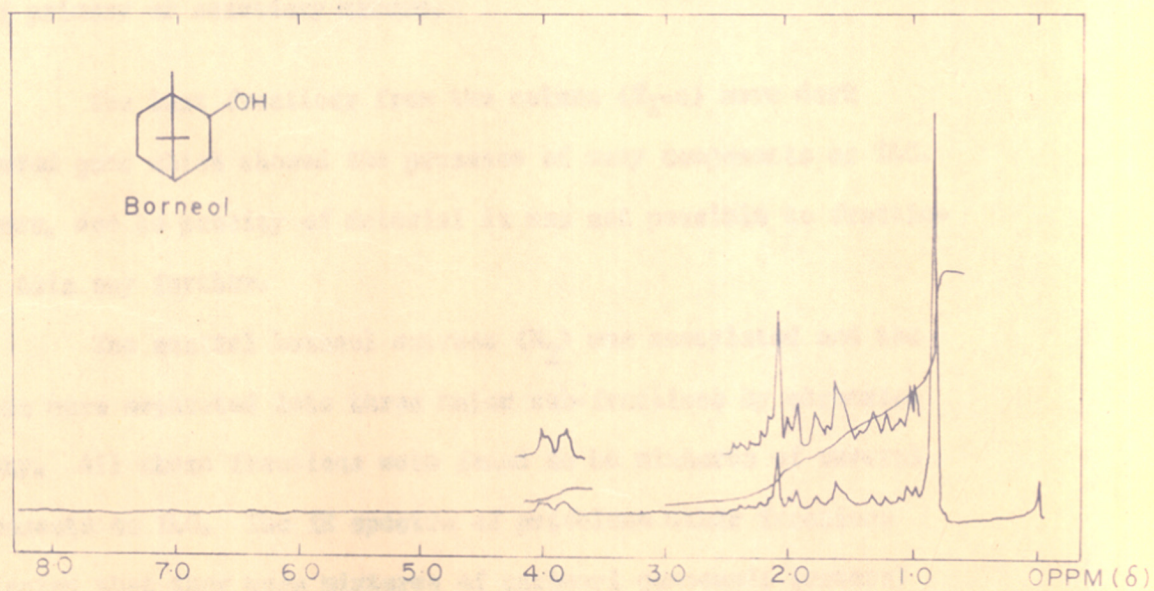
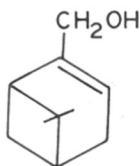


FIG. - X

the distillate showed three spots on TLC. It was possible however to identify the intermediate spot as myrtenol (49) with the help of comparative TLC and VPC (Fig.XI). The myrtenol peak had the highest retention time in VPC. By using the same technique it was possible to establish the existence of borneol in this fraction. It was not possible however to resolve the mixture to get the pure components in sufficient amounts for chemical work. The third component could not be identified.



MYRTENOL (49)

On acetylation with pyridine and acetic anhydride the alcohols in this fraction were all converted into acetates as judged by mobility on TLC plates and the absence of any hydroxyl band in the IR spectra indicating that all the alcohols in this fraction may be of primary or secondary nature.

The last fractions from the column (N_1-c) were dark coloured gums which showed the presence of many components on TLC. However, due to paucity of material it was not possible to fractionate this any further.

The neutral butanol extract (N_2) was acetylated and the esters were separated into three major sub-fractions by chromatography. All these fractions were found to be mixtures of several components on TLC. The IR spectra of petroleum ether fractions indicated that they were mixtures of carbonyl compounds (esters) ($\nu_{\max}$ 1725 and 1675 cm^{-1}) with no hydroxyl group.

The slower moving sub-fractions showed strong IR bands

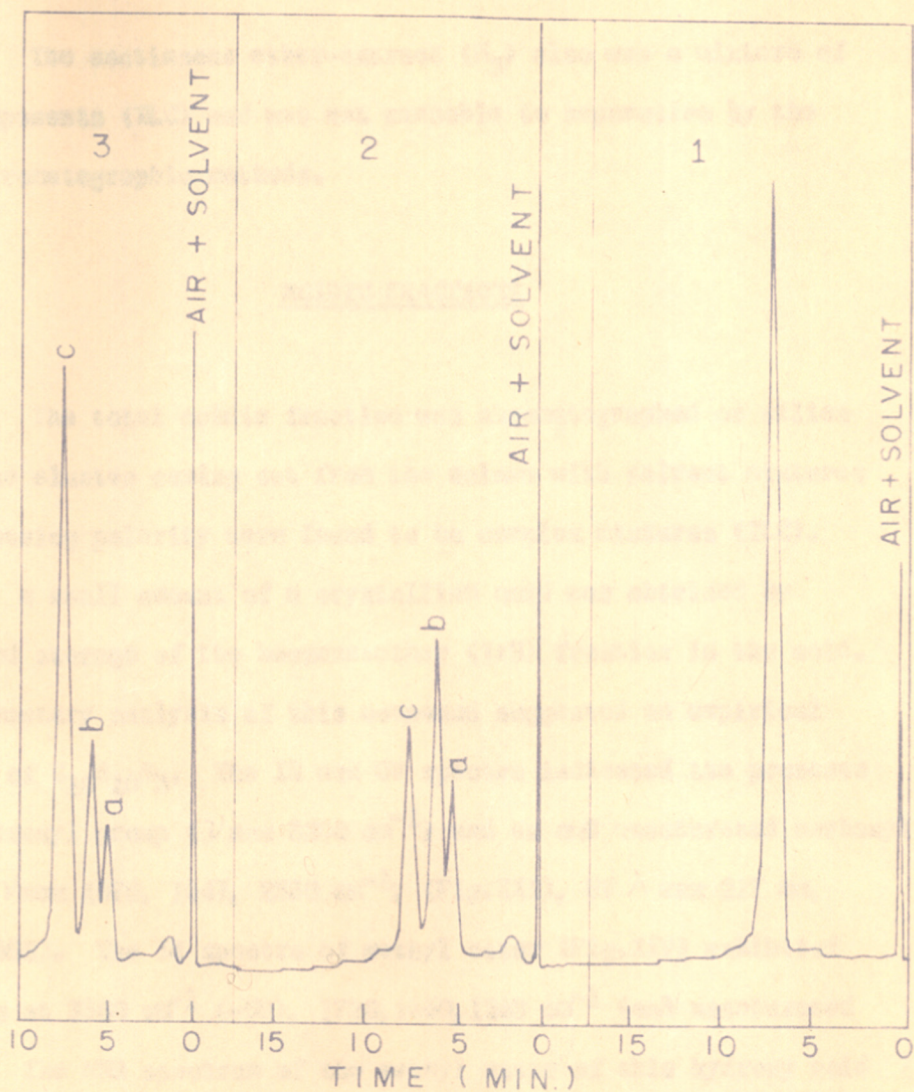


FIG. XI

IDENTIFICATION OF MYRTENOL(49) IN 100% ETHER FRACTION
 BY V. P. C. (Temp. 160°C , 100: 2 AZELAIC ACID POLYESTER COLUMN
 CARRIER GAS - HYDROGEN, FLOW RATE 10 cc / 9 Seconds.)

- ① MYRTENOL
- ② 100% ETHER FRACTION
- ③ MYRTENOL + 100% ETHER FRACTION.

in the hydroxyl region ($\nu_{\max}$ 3430 cm^{-1}) along with the carbonyl bands ($\nu_{\max}$ 1720 and 1650 cm^{-1}). However, it was not possible to resolve these sub-fractions any further into individual components.

The continuous ether-extract (N_3) also was a mixture of many components (TLC) and was not amenable to separation by the usual chromatographic methods.

ACIDIC FRACTIONS

The total acidic fraction was chromatographed on silica gel. The eluates coming out from the column with solvent mixtures of increasing polarity were found to be complex mixtures (TLC). However, a small amount of a crystalline acid was obtained by prolonged storage of the benzene:ether (1:3) fraction in the cold. The elementary analysis of this compound suggested an empirical formula of $\text{C}_{10}\text{H}_{16}\text{O}_3$. The IR and UV spectra indicated the presence of a hydroxyl group ($\nu_{\max}$ 3290 cm^{-1}) and an α,β unsaturated carboxyl group ($\nu_{\max}$ 1676, 1647, 2550 cm^{-1} ; (Fig.XII), UV $\lambda_{\max}$ 217 m μ , $\epsilon_{\max}$ 9653). The IR spectra of methyl ester (Fig.XIV) exhibited IR bands at 3500 cm^{-1} (-OH), 1720, 1660, 1263 cm^{-1} (α,β unsaturated ester). The NMR spectrum of the methyl ester of this hydroxy acid (Fig.XIII) exhibited the presence of a singlet of six protons at δ 8.82 τ , (two -C-CH₃), an olefinic proton at 3.03 τ and a carbomethoxy grouping at δ 6.33 τ . From the physical properties, the spectral data and the observed resistance towards acetylation, this compound was suspected to be oleuropeic acid (Mechoulam et al, 1962). The identity of the compound with oleuropeic acid (50) was finally established by spectral comparison (Fig.XII) and mixed melting point determination

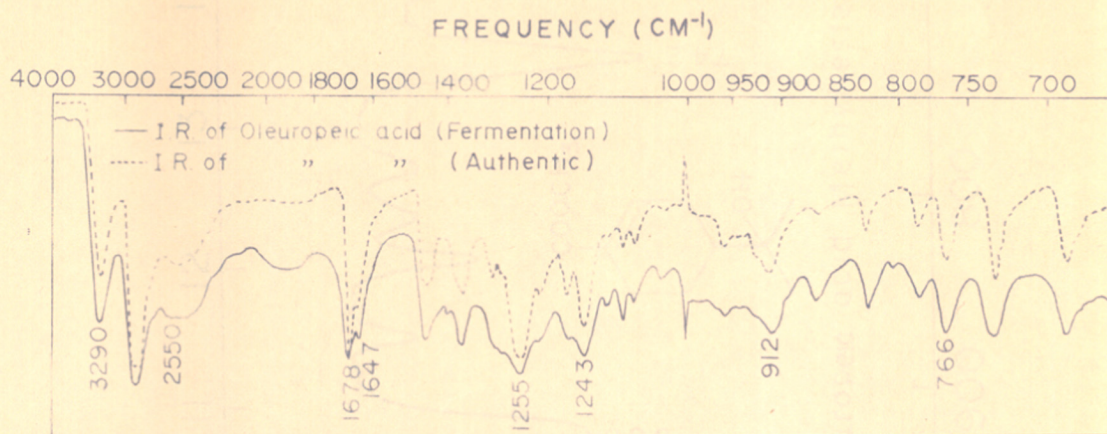


FIG. XII

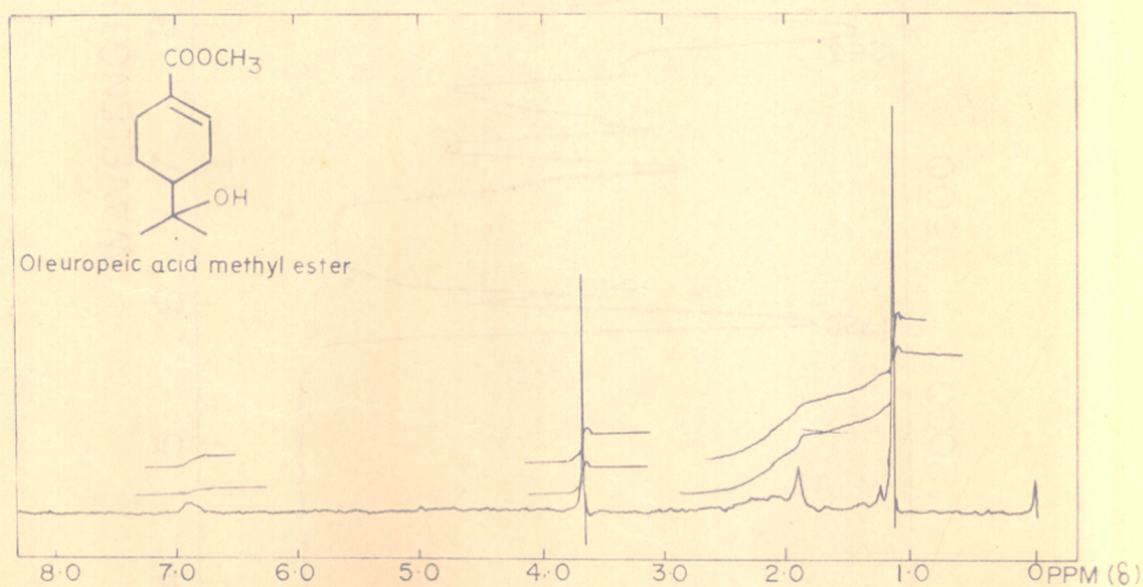


FIG. XIII

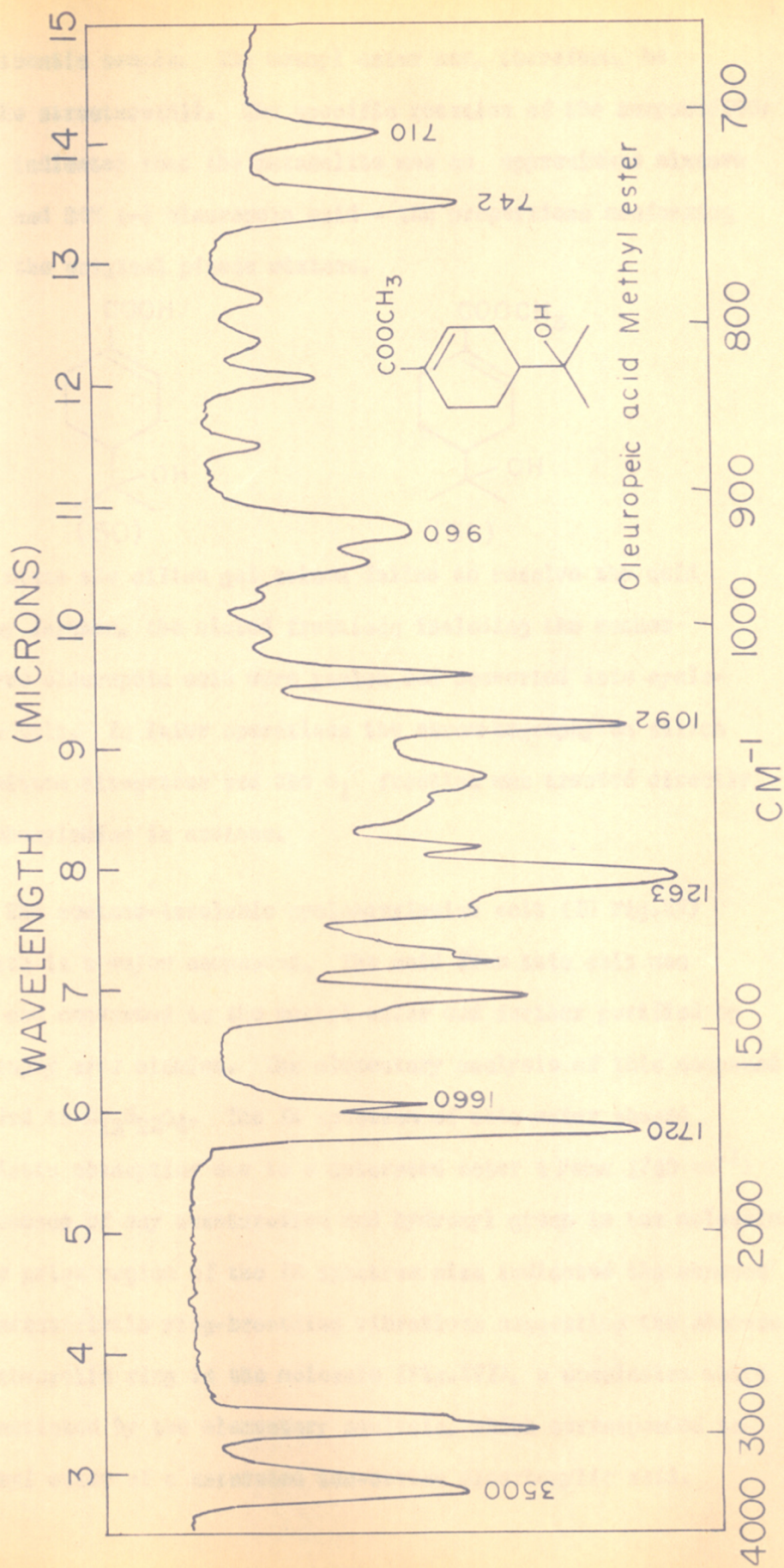
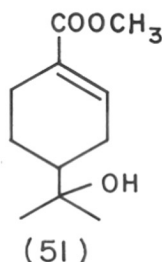
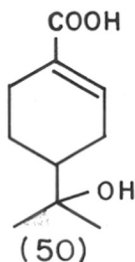


FIG. XIV

with an authentic sample. The methyl ester may, therefore, be assigned the structure(51). The specific rotation of the compound(50) $[\alpha]_D^{25} +46.7^\circ$ indicated that the metabolite was an approximate mixture of 70% (+) and 30% (-) oleuropeic acid - the proportions conforming closely to the original pinene mixture.



Since the silica gel column failed to resolve the acid mixture any further, the eluted fractions including the mother liquors from oleuropeic acid were pooled and converted into cyclohexylamine salt. In later operations the chromatography on silica gel was omitted altogether and the A_1 fraction was treated directly with cyclohexylamine in acetone.

The acetone-insoluble cyclohexylamine salt (IR Fig.XV) carried with it a major component. The acid from this salt was liberated and converted to the methyl ester and further purified by chromatography over alumina. The elementary analysis of this compound corresponded to $C_{12}H_{22}O_4$. The IR spectrum of this ester showed characteristic absorption due to a saturated ester ($\nu_{\max}$ 1740 cm^{-1}) and the absence of any unsaturation and hydroxyl group in the molecule. The finger print region of the IR spectrum also indicated the absence of any characteristic ring-breathing vibrations suggesting the absence of any carbocyclic ring in the molecule (Fig.XVI), a conclusion which was substantiated by the elementary analysis, which corresponded to the dimethyl ester of a saturated ten-carbon dicarboxylic acid.

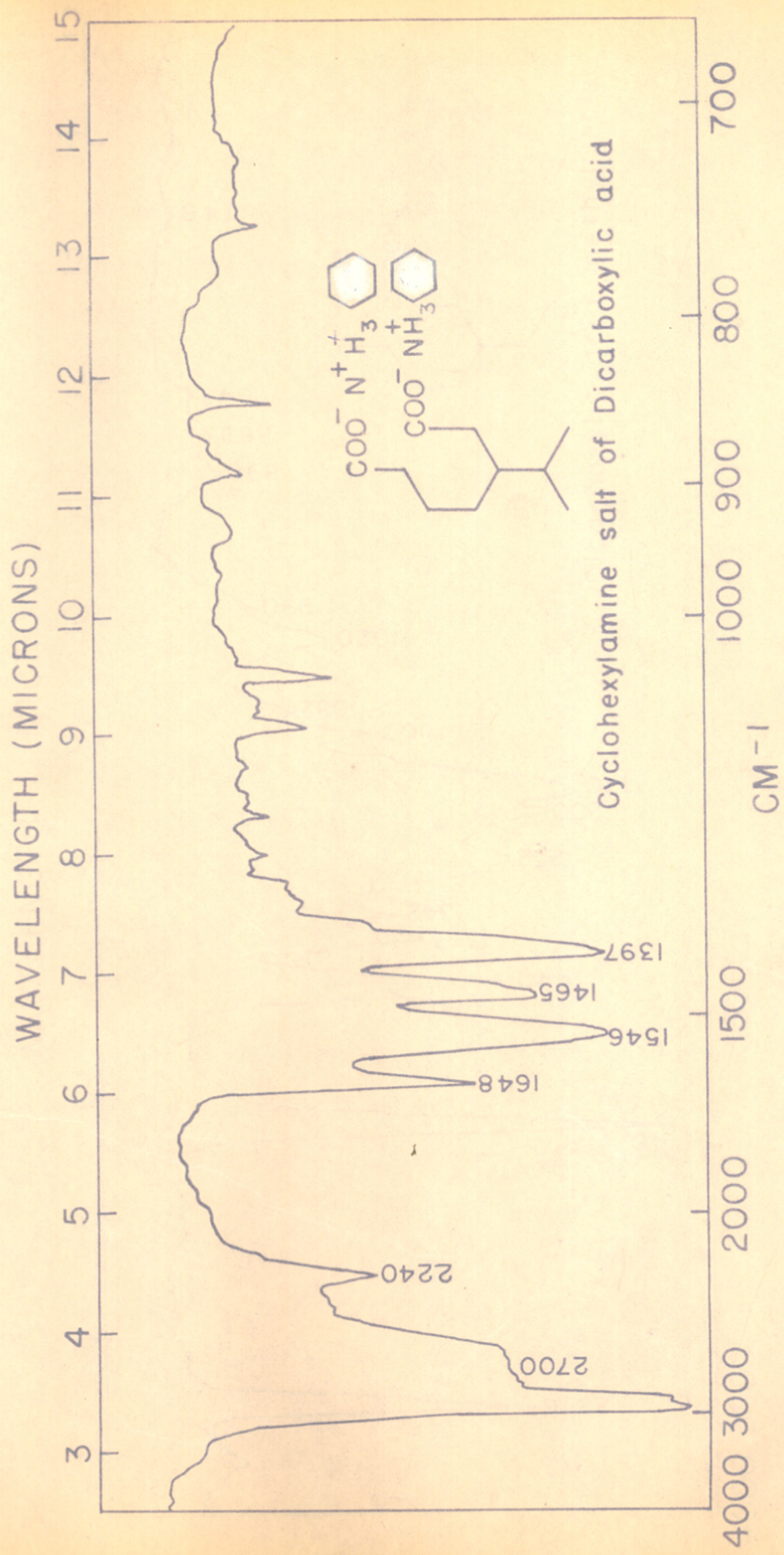


FIG. XV

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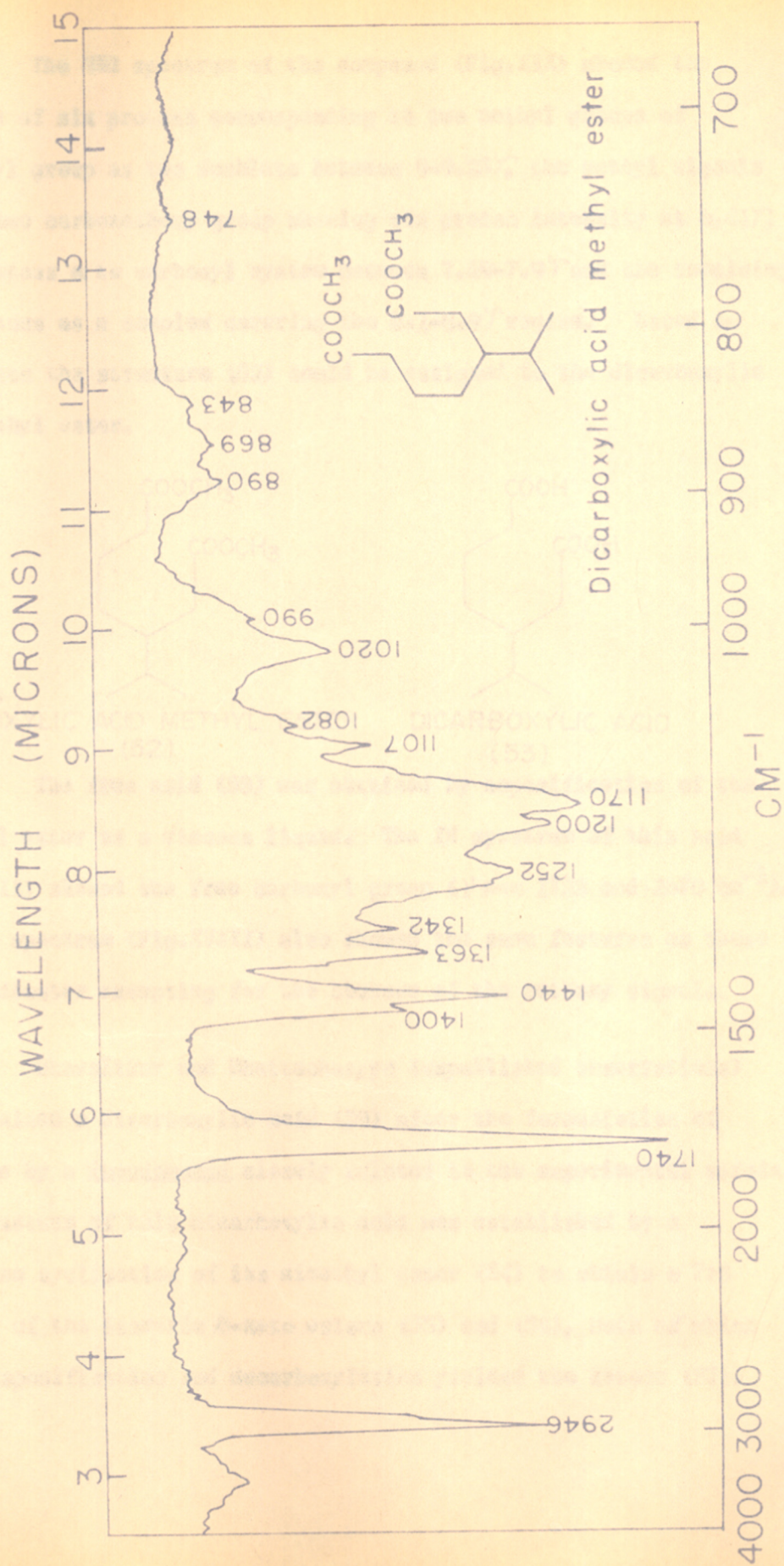
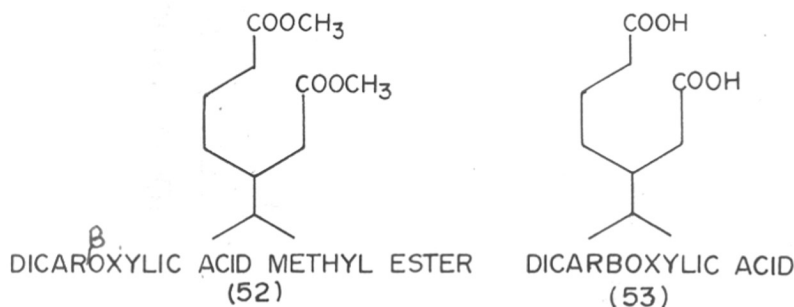


FIG. XVI

The NMR spectrum of the compound (Fig.XIX) showed the presence of six protons corresponding to two methyl groups of isopropyl group as two doublets between 9-9.25 τ , the methyl signals of the two carbomethoxy group showing six proton intensity at 6.41 τ , four protons α to carbonyl system between 7.69-7.9 τ and the remaining six protons as a complex covering the 8.1-8.9 τ region. Based on these data the structure (52) could be assigned to the dicarboxylic acid methyl ester.



The free acid (53) was obtained by saponification of the dimethyl ester as a viscous liquid. The IR spectrum of this acid (Fig.XVII) showed the free carboxyl group (ν max 1722 and 2670 cm^{-1}). The NMR spectrum (Fig.XVIII) also showed the same features as those of the diester excepting for the absence of the methoxy signals.

Dhavalikar and Bhattacharyya (unpublished observations) had obtained a dicarboxylic acid (39) after the fermentation of limonene by a *Pseudomonas* closely related to the experimental strain. The structure of this dicarboxylic acid was established by a Dieckmann cyclization of its dimethyl ester (54) to obtain a 7:3 mixture of the isomeric β -keto esters (55) and (56), both of which after saponification and decarboxylation yielded the ketone (57).

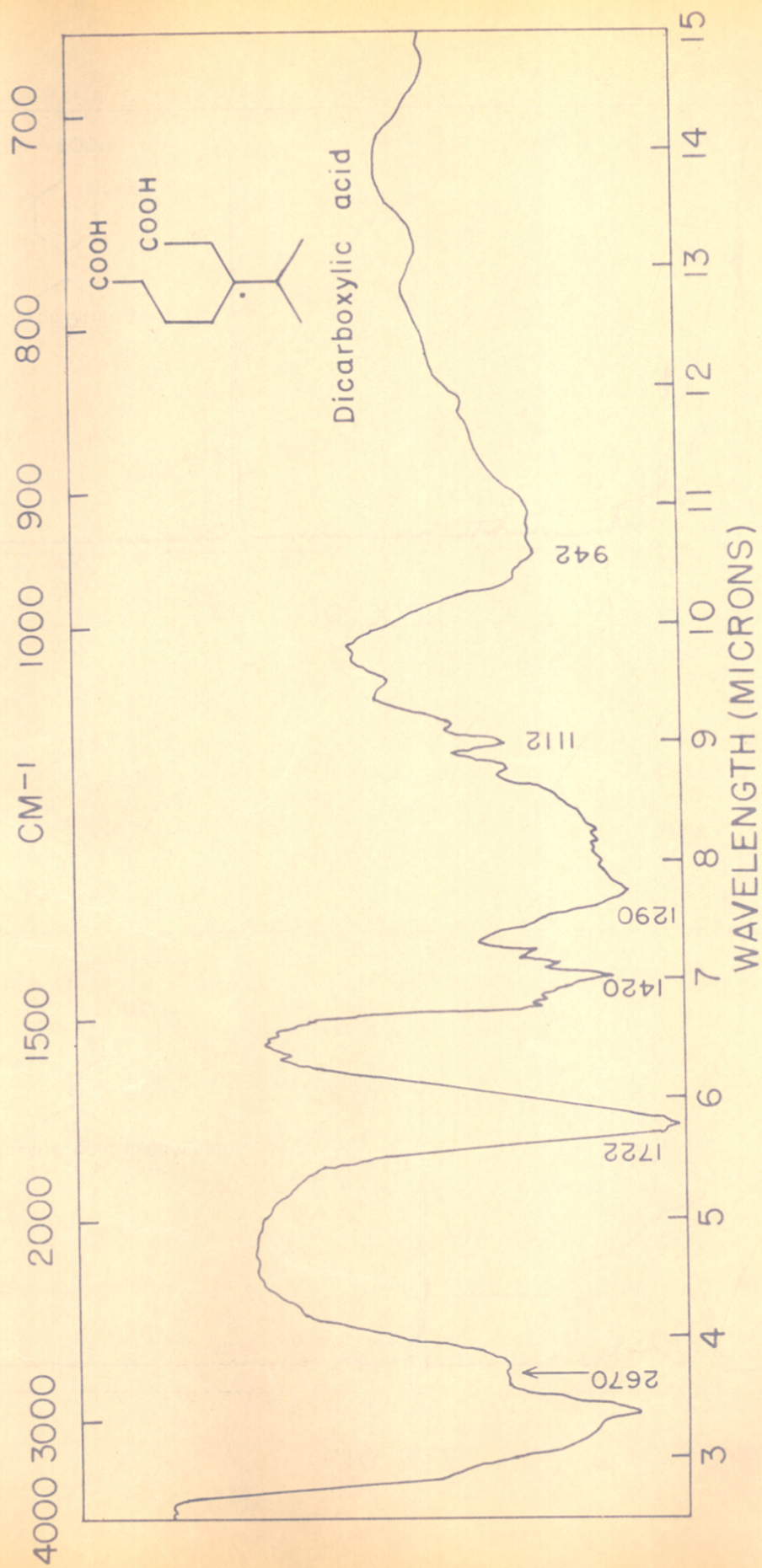


FIG. XVII

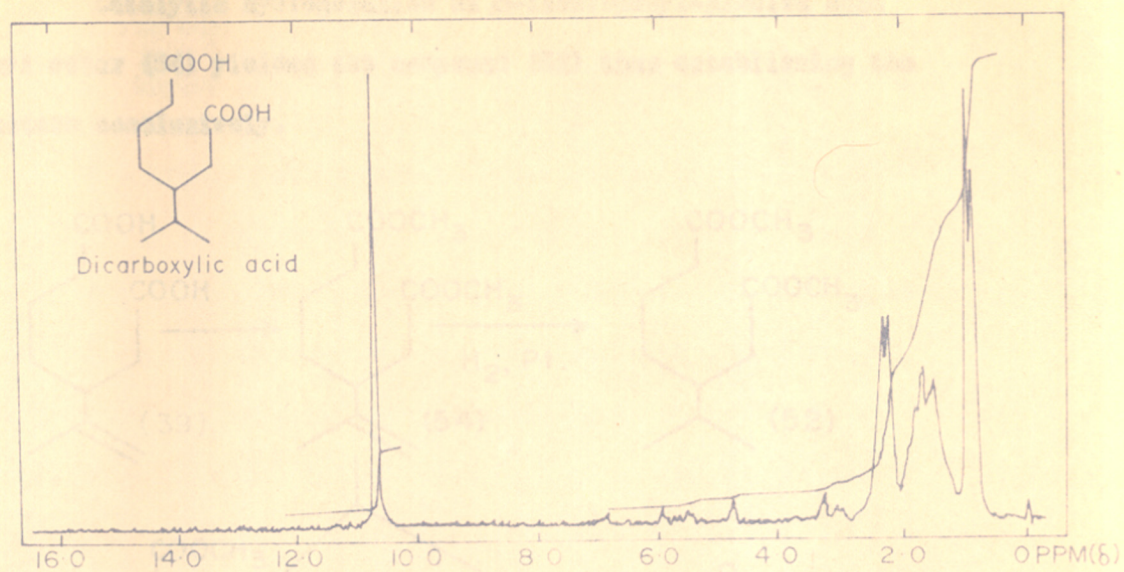


FIG. XVIII

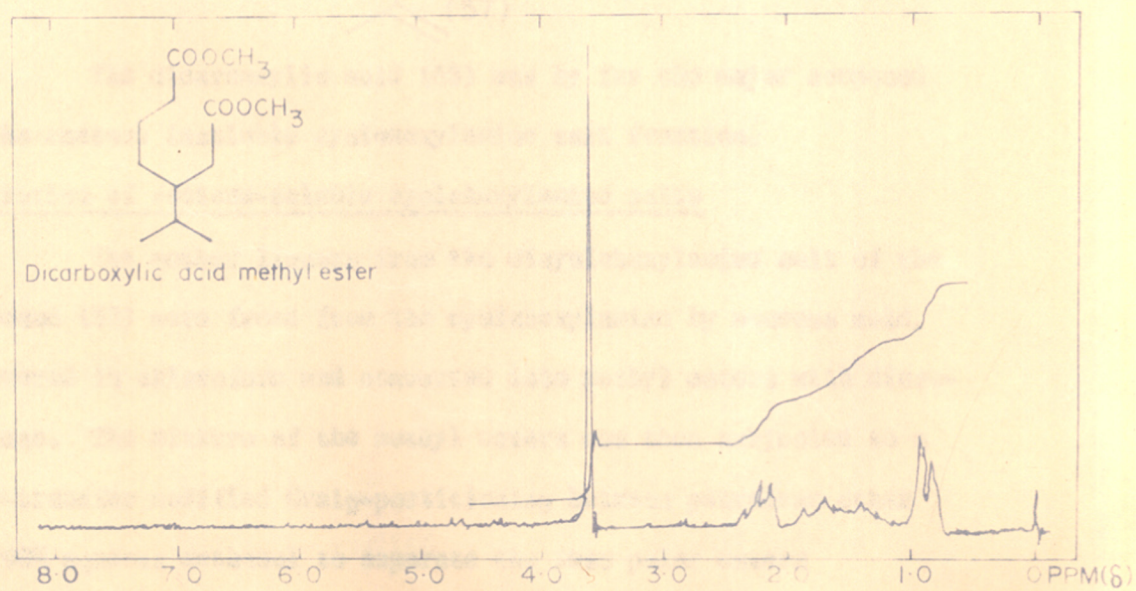
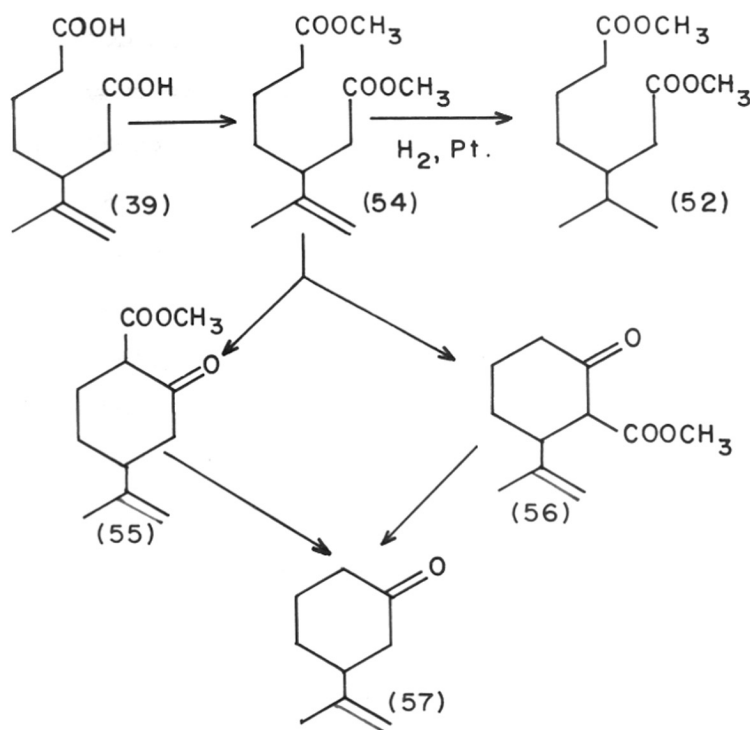


FIG. XIX

Catalytic hydrogenation of β -isopropenyl-pimelic acid methyl ester (54) yielded the compound (52) thus establishing the structure conclusively.



The dicarboxylic acid (53) was by far the major compound in the acetone insoluble cyclohexylamine salt fraction.

Separation of acetone-soluble cyclohexylamine salts

The mother liquors from the dicyclohexylamine salt of the compound (53) were freed from the cyclohexylamine by aqueous acid, recovered in chloroform and converted into methyl esters with diazomethane. The mixture of the methyl esters was then subjected to a four-transfer modified Craig-partitioning between petroleum ether and 90% aqueous methanol to separate the less polar esters (Fraction A_{1a}) and the more polar ones (Fraction A_{1b}). These fractions were then processed separately.

Fraction A_{1a}: The less polar fraction on fractional distillation yielded a lower boiling sub-fraction b.p. 100°-130°C/1.5 mm (bath temp.) and a higher boiling fraction b.p. >160°/1.5 mm (bath temp.).

The higher boiling sub-fraction was purified by repeated distillation ~~in vacuo~~ and by chromatography of the final distillate on an alumina column (Grade I). The total fraction from the column appeared to be homogeneous and was distilled. The distillate was saponified and the free acid was separated from acetonitrile as a low melting solid which analysed for C₂₀H₄₀O₂. The IR spectra of the methyl ester (Fig.XX) exhibited ester band ($\nu_{\max}$ 1749 cm⁻¹) and a long methylene chain ($\nu_{\max}$ 725 cm⁻¹), the absence of a double bond, carbocyclic ring or any other distinguishable functional groups and a close similarity to those of fatty acids. The NMR spectrum (not shown) substantiated the conclusion that this compound was a straight chain fatty acid. No further attempts were made to determine the exact structure of this compound.

The lower boiling sub-fraction from A_{1a} gave an elementary analysis corresponding to the methyl ester of a ten-carbon monocarboxylic acid but was shown to be a complex mixture by VPC. This was further purified by chromatography over alumina and collecting the petroleum ether fraction carefully. The faster moving component showed a single spot on TLC and after purification by vacuum distillation analysed for C₁₁H₁₈O₂(A_{1a}-1). The IR spectrum was rather unexpectedly complex showing both saturated and unsaturated ester bands ($\nu_{\max}$ 1735 and 1713 cm⁻¹). The free acid obtained by saponification also exhibited a similar complexity. The NMR spectra of both the ester and the free acid were also complex indicating that

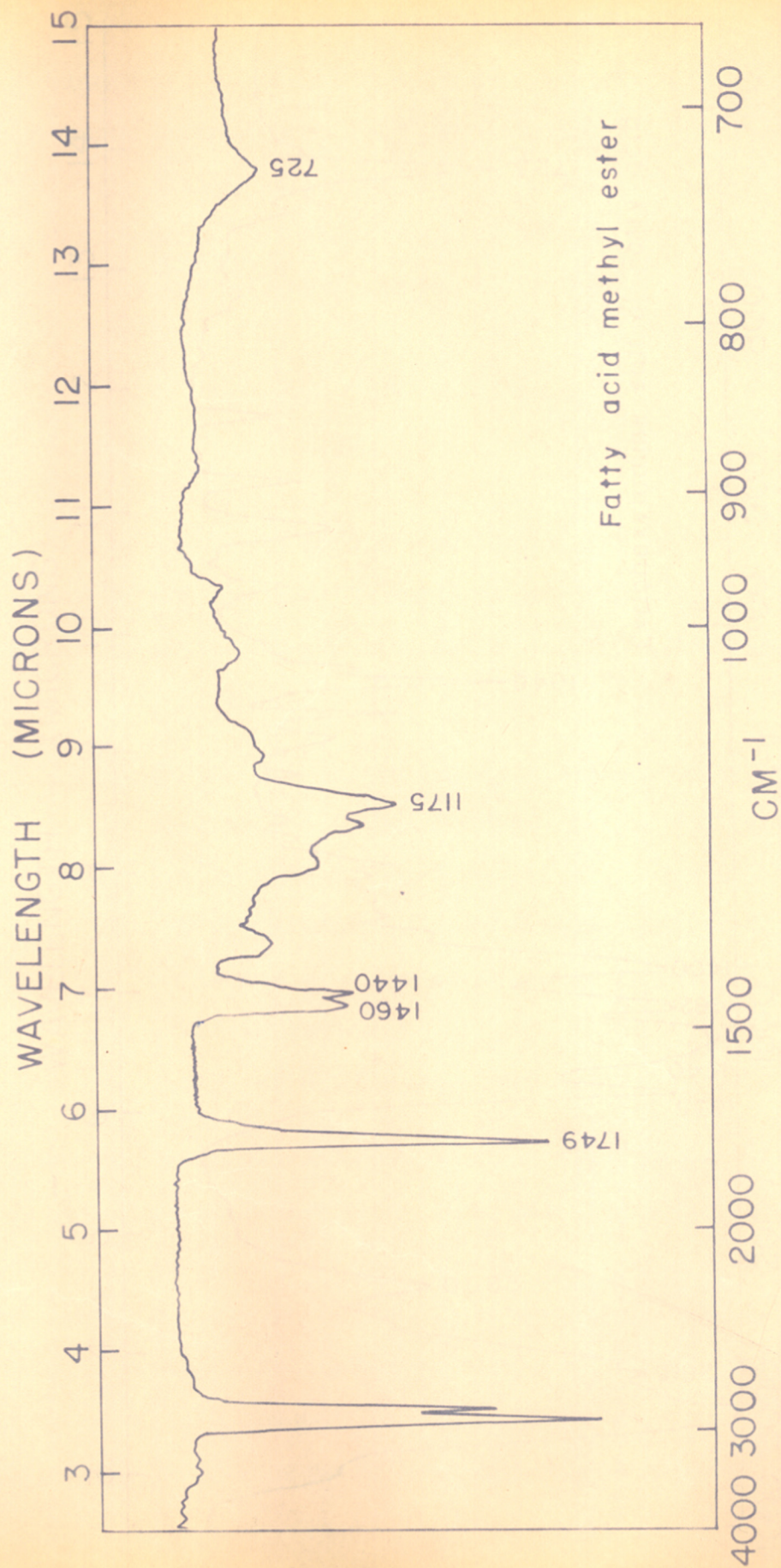


FIG. XX

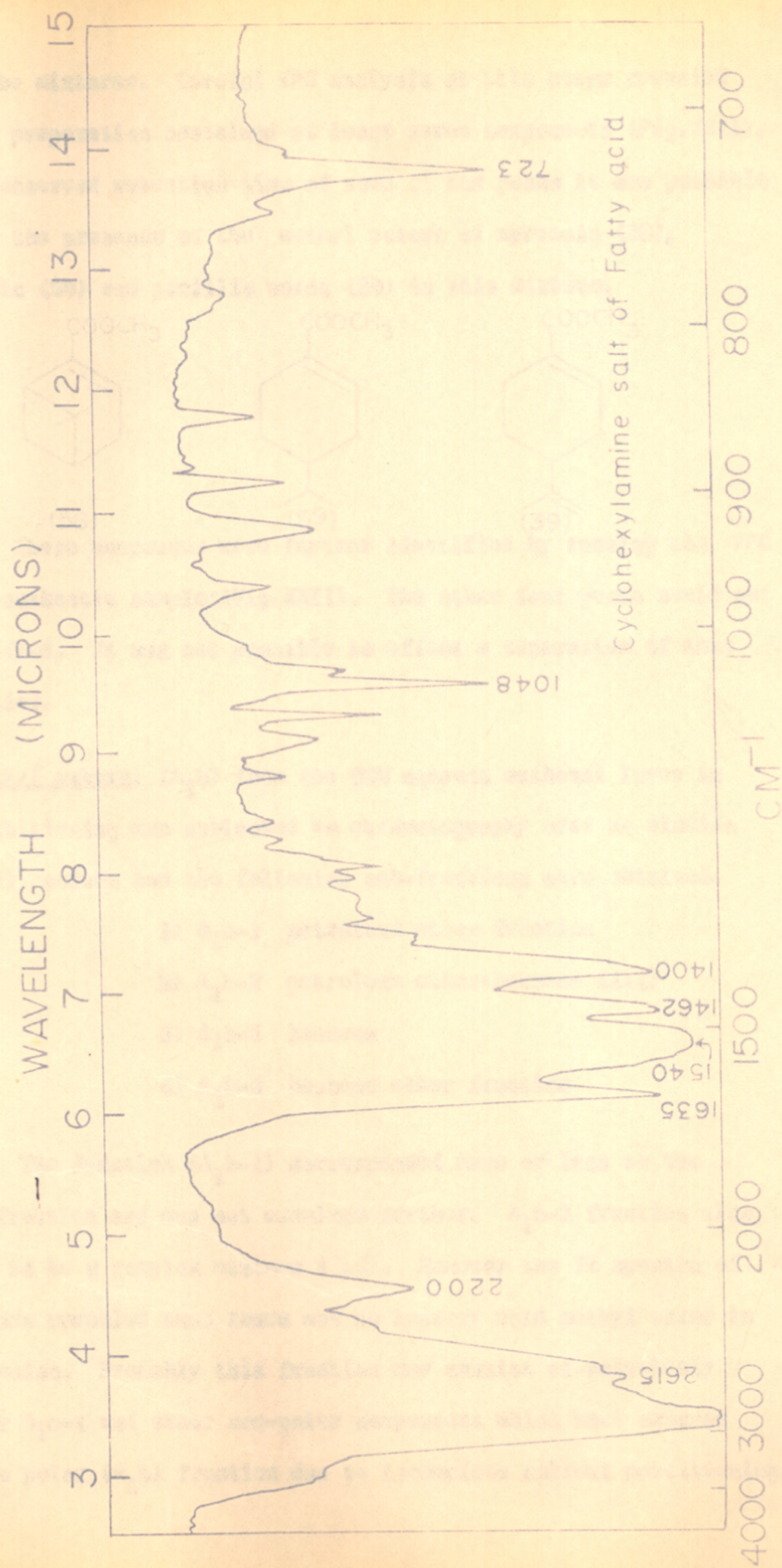
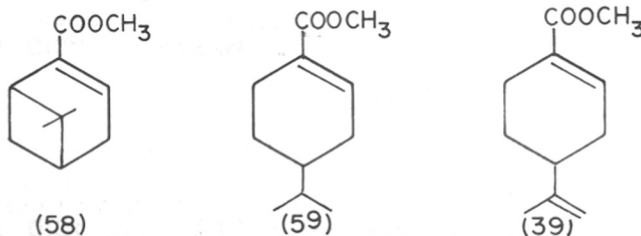


FIG. XXI

they may be mixtures. Careful VPC analysis at this stage revealed that this preparation contained at least seven components (Fig. XXII). From the observed retention time of some of the peaks it was possible to deduce the presence of the methyl esters of myrtenic (58), phellandric (59) and perillic acids (39) in this mixture.



These compounds were further identified by running the VPC with the authentic samples (Fig. XXII). The other four peaks could not be identified. It was not possible to effect a separation of this sub-fraction.

Polar methyl esters: (A₁b) from the 90% aqueous methanol layer in Craig-partitioning was subjected to chromatography over an alumina (Grade II) column and the following sub-fractions were obtained.

- 1) A₁b-1 petroleum ether fraction
- 2) A₁b-2 petroleum ether:benzene (1:1)
- 3) A₁b-3 benzene
- 4) A₁b-4 benzene ether fraction

The fraction (A₁b-1) corresponded more or less to the (A₁a-1) fraction and was not examined further. A₁b-2 fraction also appeared to be a complex mixture (TLC). However the IR spectra of the mixture revealed that there was no hydroxy acid methyl ester in this fraction. Probably this fraction may consist of relatively non-polar A₁a-1 and other non-polar components which have crossed over into polar (A₁b) fraction due to incomplete solvent partitioning.

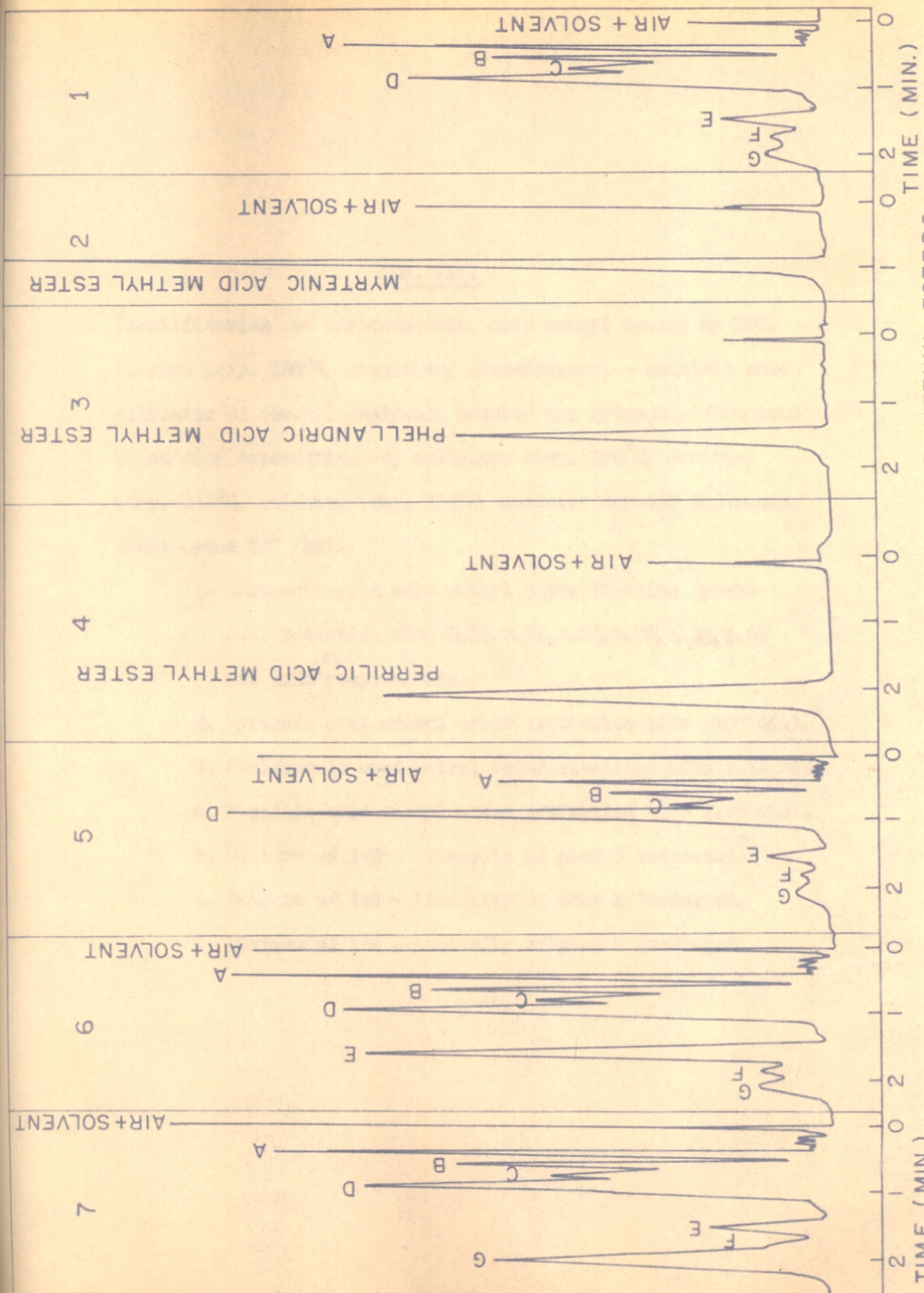


FIG. XXII - IDENTIFICATION OF MONOCARBOXYLIC ACID METHYL ESTERS

(DETAILS - SEE NEXT PAGE)

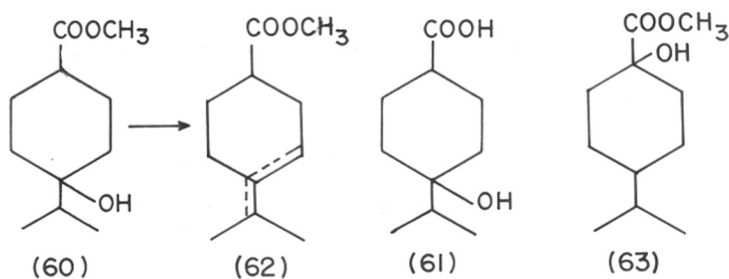
FIG. XXII

Identification of monocarboxylic acid methyl esters by VPC.
(Column temp. 123°C, stationary phase/support - succinic acid polyester of diethyleneglycol; carrier gas hydrogen, flow rate 30 cc/min; Attenuation -4, collector temp. 200°C, detector temp. 230°C, injector temp. 190°C; detector current 200 m.amp. Chart speed 30" /hr).

1. Monocarboxylic acid methyl ester fraction (peaks A-G, retention time 0.35, 0.51, 0.59, 0.76, 1.34, 1.65 min and 1.85/ respectively.)
2. Myrtonic acid methyl ester (retention time 0.76 min).
3. Phellandric acid methyl ester (retention time 1.34 min).
4. Perillic acid methyl ester (retention time 1.85 min).
5. Mixture of 1+2 - intensity of peak D increased.
6. Mixture of 1+3 - intensity of peak E increased.
7. Mixture of 1+4 - intensity of peak G increased.

The third sub-fraction (4b-3) came through the column with considerable trailing and appeared to be 90% pure by TLC. On working up this fraction a liquid hydroxyester was obtained which showed characteristic IR bands of a saturated hydroxyester (ν_{max} 3500, 1167 and 1732 cm^{-1}) and the absence of any double bond (Fig. XXIII). The compound could not be acetylated with pyridine and acetic anhydride under the usual conditions, indicating that the hydroxyl group was tertiary in nature. The NMR spectrum (Fig. XXIV) revealed an isopropyl group (split doublet of six proton intensity at 9.14τ , J 6.5 cps) a carbomethoxy group (a singlet of three proton intensity (6.39τ)). The complex signal between 7.49 - 7.75τ may be due to ^{the}proton alpha to the carboxyl group. The hydroxyl proton appeared as a band at 8.87τ (↓) which is reduced in intensity by equilibration with D_2O .

The spectral data suggested the structure (60) for this compound and (61) for the corresponding acid. Dehydration with



thionyl chloride in pyridine gave an unsaturated ester presumably (62) (λ_{max} 212 m μ , ϵ_{max} 2693 indicating that the hydroxy group was not in the vicinity of carboxyl group as in (63).

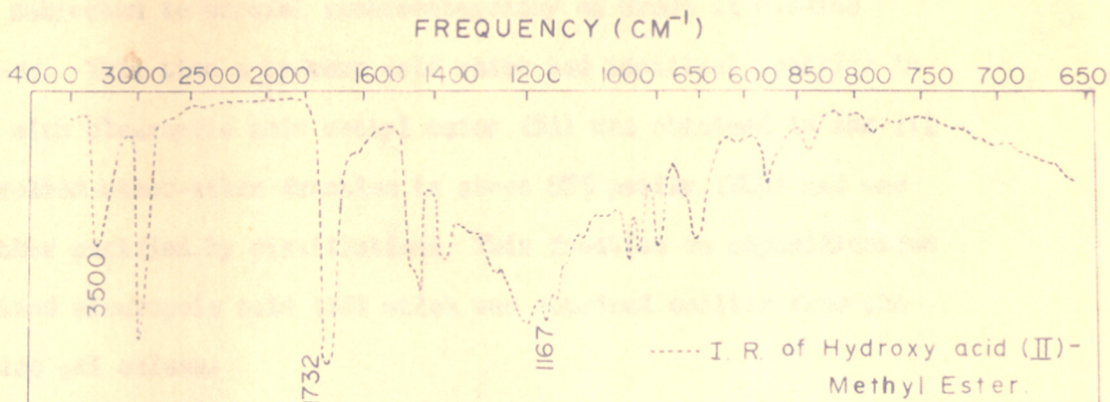


FIG. XXIII

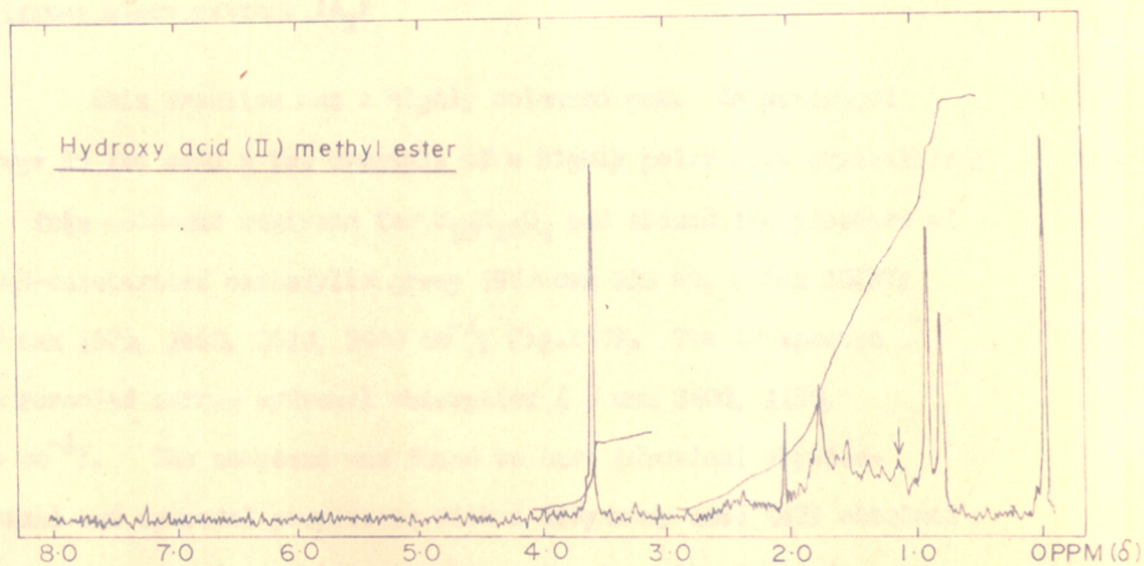


FIG. XXIV

The benzene-ether (1:1) fraction (A_{1b-4}) was a major fraction and showed mainly two spots on TLC. This sub-fraction was subjected to careful rechromatography on Grade II alumina column. This time a hydroxy acid which had identical mobility in TLC with oleuropeic acid methyl ester (51) was obtained in the 1:1 petroleum ether-ether fraction in about 85% purity (TLC) and was further purified by distillation. This fraction on saponification yielded oleuropeic acid (50) which was obtained earlier from the silica gel column.

Butanol Fraction (A_2)

This fraction was methylated with diazomethane and chromatographed over Grade II alumina. The fraction eluting with 1:2 and 1:1 ether-petroleum ether mixture contained a major compound which was also identified as oleuropeic acid methyl ester (51).

Continuous ether extract (A_3)

This fraction was a highly coloured gum. On prolonged storage in the cold a few crystals of a highly polar acid crystallised out. This compound analysed for $C_{10}H_{16}O_4$ and showed the presence of an α,β -unsaturated carboxylic group ($UV \lambda_{max}$ 218 m μ , ϵ_{max} 10737; IR ν_{max} 1676, 1660, 2610, 2940 cm^{-1} ; Fig. XXV). The IR spectra also revealed strong hydroxyl absorption (ν_{max} 3400, 1150, 1056 cm^{-1}). The compound was found to have identical physico-chemical and spectral properties with a dihydroxy acid (42) obtained from the fermentation of limonene by a closely related Pseudomonas (Dhavalikar and Bhattacharyya- unpublished observations). These

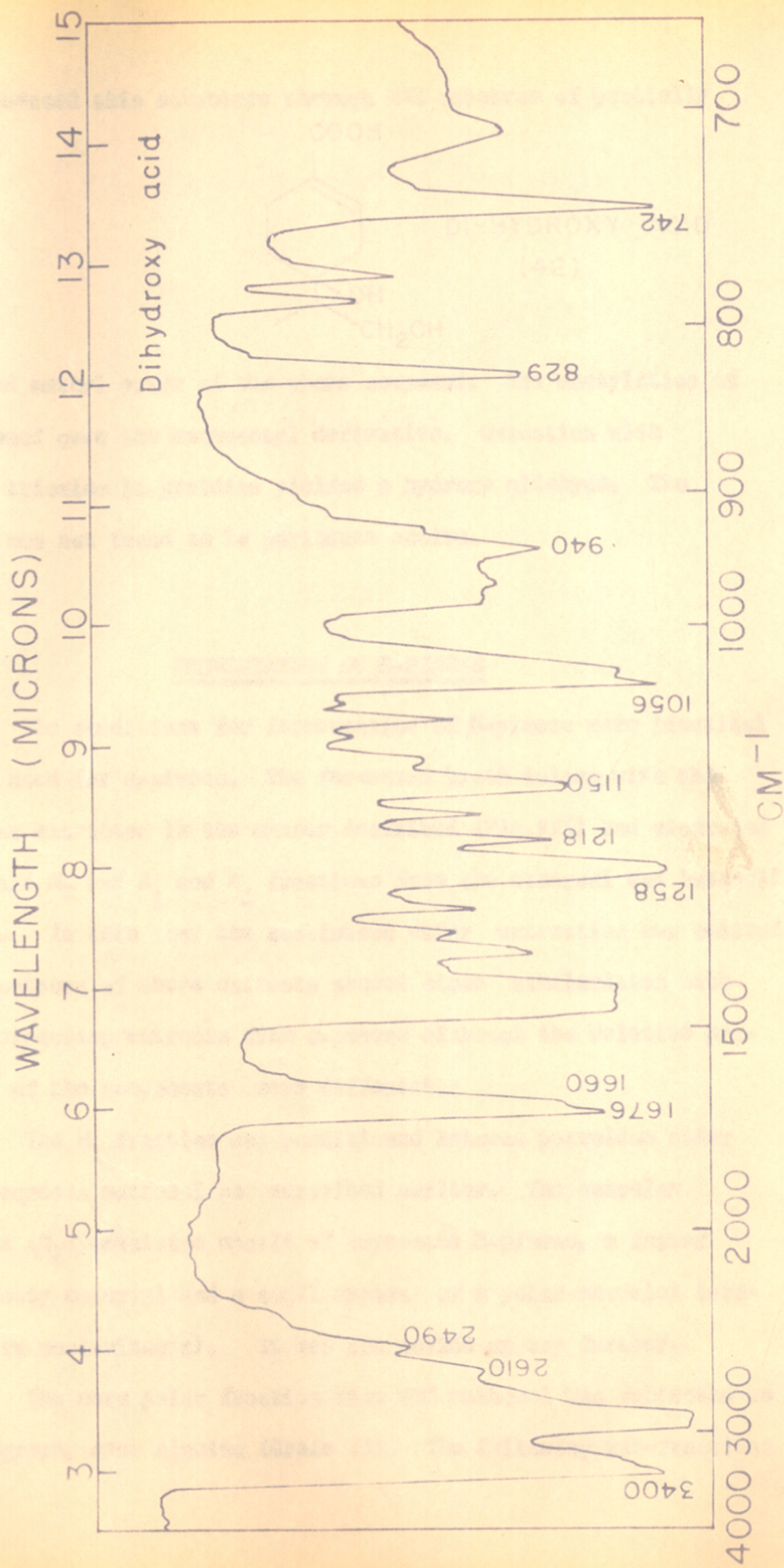
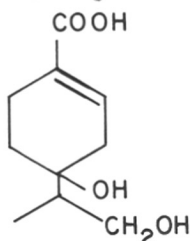


FIG. XXXV

workers deduced this structure through NMR spectrum of partially



DI-HYDROXY ACID
(42)

acetylated methyl ester of the above compound. The acetylation of the compound gave the monoacetyl derivative. Oxidation with chromium trioxide in pyridine yielded a hydroxy aldehyde. The compound was not found to be periodate active.

FERMENTATION OF β -PINENE

The conditions for fermentation of β -pinene were identical with those used for α -pinene. The fermented broth (along with the cells) was extracted in the manner described (Fig.VII) and separated into N_1 and N_2 and A_1 and A_2 fractions from the ethereal and butanolic extracts. In this case the continuous ether extraction was omitted. The TLC pattern of these extracts showed close similarities with the corresponding extracts from α -pinene although the relative proportions of the components were different.

The N_1 fraction was partitioned between petroleum ether and 90% aqueous methanol as described earlier. The nonpolar component (N_{1a}) consisted mostly of unreacted β -pinene, a faster moving fatty material and a small amount of a polar material (presumably as contaminants). It was not worked up any further.

The more polar fraction from 90% methanol was subjected to chromatography over alumina (Grade II). The following sub-fractions

were collected:

- 1) N_1b-1 petroleum ether
- 2) N_1b-2 ether + petroleum ether (1:3)
- 3) N_1b-3 ether + petroleum ether (1:1) and ether
- 4) N_1b-4 ether + ether-methanol

The first sub fraction (N_1b-1) was found to be a nonpolar contaminant coming into 90% methanol layer.

The fractions (N_1b-2 and N_1b-3) on working yielded mainly l-borneol (47), $[\alpha]_D -17.9^\circ$ identified by its physico-chemical properties and comparison of IR spectra with d(+) borneol.

The fraction (N_1b-3) was demonstrated by TLC to contain borneol (47). The TLC pattern of this fraction was similar to the 100% ether fraction from α -pinene neutral products (N_1d). The nature of these compounds could not be established.

The neutral fraction from butanol (N_2) also showed a number of spots on TLC which could not be isolated and characterised.

Acid fractions (A_1)

In the case of β -pinene fermentation, the acidic fraction directly deposited crystals after removal of solvent and cooling. These crystals were purified and identified as (-) oleuropeic acid (50) by comparative IR spectroscopy with (+) oleuropeic acid obtained by fermentation of α -pinene. It should be mentioned that the rotation $[\alpha]_D -114$ of this compound is very close to the rotation of pure leaverotatory oleuropeic acid (lit $[\alpha]_D -125$, Mechoulam et al, 1962). There was no depression of m.p. by mixing

it with an authentic sample from above workers. Considerable amount of this oleuropeic acid is obtained from the A_1 fraction.

From the mother liquors of A_1 fraction after removal of oleuropeic acid, the dicarboxylic acid (53) was separated by treatment with cyclohexylamine in acetone. This dicyclohexylamine salt of the acid as well as the dimethyl ester were found to be identical with the corresponding compounds from α -pinene. Surprisingly enough, the specific rotations of the dicarboxylic acid irrespective of its origin from α or β pinene were identical, $[\alpha]_D -5^\circ$.

The acidic fraction after removal of cyclohexylamine salt was worked up in the fashion described earlier. The methyl esters obtained were subjected to chromatography over Grade-II alumina. In this case the solvent partitioning step was omitted. From the column the following sub-fractions were collected.

- 1) A_{1a} - petroleum ether
- 2) A_{1b} - benzene
- 3) A_{1c} - ether
- 4) A_{1d} - methanol

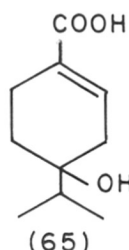
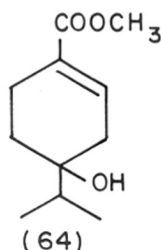
The A_{1a} -a fraction had ~~an~~ almost identical UV and IR spectra with ^{those of} the nonpolar fraction from α -pinene. This was separated into the higher boiling fatty ester and lower boiling monocarboxylic acid esters by fractional distillation. The latter fraction showed identical IR spectra with ^{those of} the corresponding fraction (A_{1a}) from α -pinene. It was not examined any further.

The A_{1b} -b fraction showed many spots on TLC. The fast moving component corresponding to the earlier fraction and smaller amount of another slower moving component which was also observed

in the (A_1 -c) fraction.

The fraction (A_1 -c) mainly consisted of two spots, one of them had R_f value identical with the methyl ester of oleuropeic acid. The other compound having a slightly higher R_f value than oleuropeic acid methyl ester was further purified by careful chromatography on alumina.

The IR spectra of this methyl ester (Fig. XXVI) indicated the presence of a tertiary hydroxyl group ($\nu_{\max}$ 3400, 1135 cm^{-1}) and an α,β unsaturated ester group ($\nu_{\max}$ 1720, 1660 and 1265 cm^{-1}). The UV spectrum of the compound also indicated the presence of an α,β unsaturated ester chromophore ($\lambda_{\max}$ 218 $m\mu$, 7550). The NMR spectra (Fig. XXVII) indicated the presence of an isopropyl side chain (a doublet of six proton intensity at 9.07 τ , 6.5 cps), four allylic protons as a diffuse signal (7.3-8 τ), one olefinic proton (diffuse signal at 3.27 τ). From the physical data the probable structure (64) was assigned to this ester and (65) to the corresponding acid.



However this material was available in quantities insufficient for further chemical studies.

In addition to the compound (64) this fraction also contained oleuropeic acid methyl ester which was further purified by careful chromatography. The IR spectrum of this was identical with that of

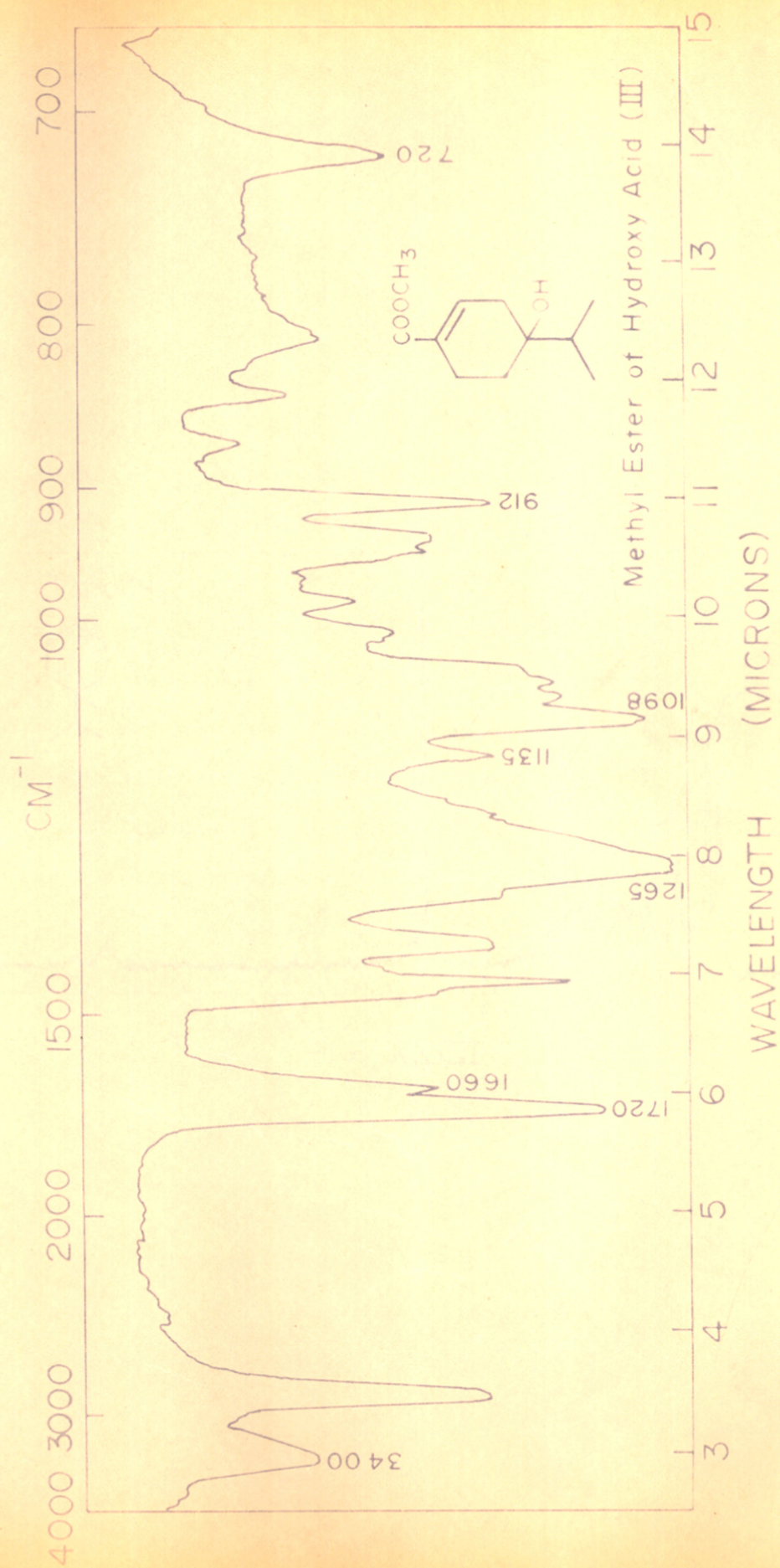


FIG XXXVI

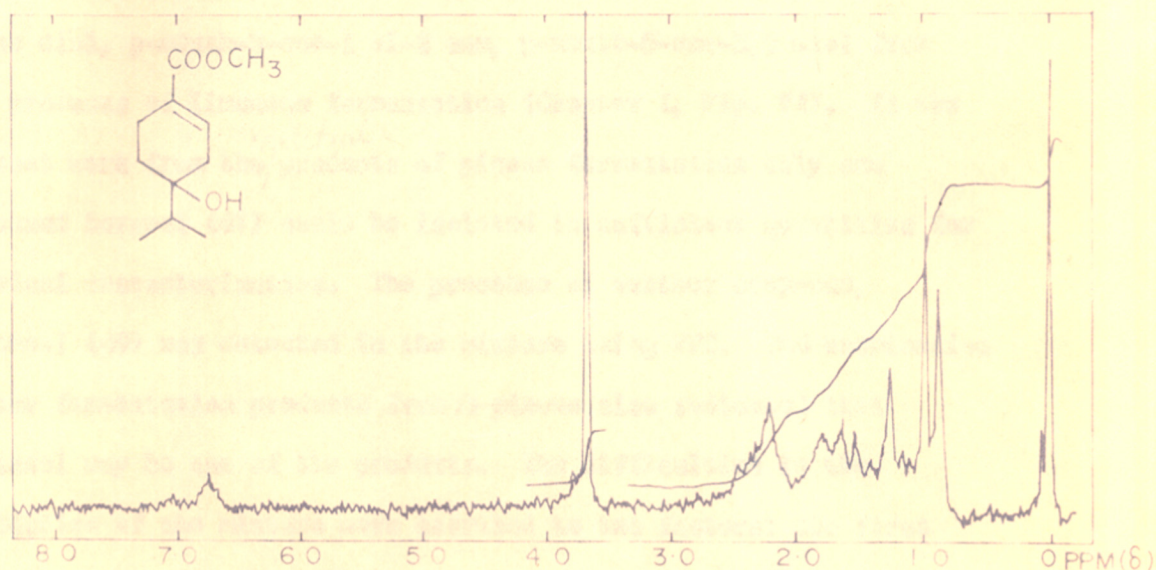


FIG. XXVII

the corresponding ester from α -pinene fermentation (Fig. XIV). Free oleuropeic acid was obtained by saponification of this ester and recrystallisation from water.

The butanol fraction (A_2) was found mainly to be a mixture of oleuropeic acid (50) and the acid (65) as revealed by comparative TLC of the methyl esters.

Bhavalikar and Bhattacharyya (1964) were able to separate at least seven neutral compounds characterized as carvone, dihydrocarvone, carveol, p-menth-8-ene-1,2 cis diol, p-menth-8-ene-1,2-trans diol, p-menth-8-ene-1 ol-2 one, p-menth-6-ene-2,9 diol from the products of limonene fermentation (Chapter I, Fig. VI). In the present work from the ^{neutral} products of pinene fermentation only one compound borneol (47) could be isolated in sufficient quantities for chemical characterization. The presence of another compound, myrtenol (49) was detected in the mixture using VPC. TLC examination of the fermentation products from β -pinene also indicated that myrtenol may be one of the products. The difficulties in the resolution of the mixture were ascribed to two factors: the first being the presence of too many components of similar polarity and boiling points, secondly the yield of these products except borneol was too low. Even the yields of borneol seldom exceeded one percent of the substrate utilised.

The acidic fractions from α - and β -pinene could be resolved somewhat more successfully and the two of the major components - oleuropeic acid (50) and β -isopropyl pimelic acid (53)- were easily isolated. Two other hydroxy acids (61) and (65) were

also isolated from the fermentation mixtures of α and β -pinene respectively, the only difference being the absence of unsaturation in the former compound. Dhavalikar and Bhattacharyya (1964) isolated perillic acid (39) Δ^8 -p-menthene-2-hydroxy-7 oic acid (40) and β -isopropenyl pimelic acid (41) from the fermentation of limonene. The strain they used was a variant of the present strain. However, as far as the TLC analysis was concerned, our strain also produced the same compounds on limonene. The only two products from the pinenes that seem to be identical are perillic acid (39) and dihydroxy acid (42).

It should be mentioned that among the neutral products the presence of α -terpineol and verbenol was specially sought. However, neither TLC nor VPC could demonstrate the presence of these compounds.

However, as will be evident in the succeeding chapter, the failure in the identification of most of the fermentation products did not hamper the attainment of the objective of the present work - the elucidation of the biochemical pathway of degradation of pinenes.

CHAPTER IV

EXPERIMENTAL

FERMENTATION OF α -PINENE

Optimum conditions

Inoculum, pH and temperature: In all the fermentations, inocula varying from 2 to 5% were used. Higher amounts resulted in a too early depletion of pinene from the medium and a small amount of inoculum resulted in ^atoo slow growth. A pH of seven was found to be the best for purposes of growth and fermentation. Lower pH_s of 6.0 was also tested but did not seem to offer any distinct advantage and the growth of the organism was slower. Higher pH resulted in clumping and lysis. All the fermentations were carried out in a constant temperature room at $25 \pm 2^{\circ}\text{C}$ on a rotary shaker (220 r.p.m.). It was found that although the organism showed a progressively higher growth rate in the range $25-37^{\circ}\text{C}$, the advantage of this higher growth rate at higher temperature was offset by very high rate of evaporation of α -pinene.

Effect of salt concentrations: After a few trials with different media, the salt medium given in Chapter III was found to be the best for the present studies.

Effect of substrate concentration: The effect of the various concentration of substrates was tested by inoculating 100 ml of mineral salt medium with two percent inoculum (of 24-hr old cells grown in the respective substrates) and adding the substrates in various concentrations ranging between 0.2 to 2%. The results

presented in Table 3 indicate two trends. In the case of α and β -pinenes growth is optimum at a concentration of 0.6%. Higher amounts of pinene were found to cause a gradual and slow decrease in growth rate. In the case of limonene and p-cymene the growth rate reached a maximum at 0.6% concentration and remained constant with higher concentrations. As a result of these observations a concentration of 0.3-0.5% of α or β -pinene was employed throughout the fermentation. Additional amount of substrate (0.3 and 0.4%) was added at 24 hr intervals to compensate for the loss due to evaporation.

TABLE 3
Effect of concentration of the substrate on
the growth of the organism

Concentration	α -pinene	β -pinene	limonene	p-cymene
nil	29	33	29	31
0.2 ml	206, 218	200, 209	310, 298	310, 296
0.4 ml	315, 335	320, 311	360, 365	390, 395
0.6 ml	370, 380	385, 377	385, 395	395, 385
0.8 ml	340, 355	349, 352	406, 389	395, 380
1 ml	318, 326	309, 323	390, 389	385, 385
1.5 ml	300, 315	296, 305	390, 390	385, 390
2.0 ml	260, 268	250, 257	390, 387	380, 386

The accumulation of products as a function of time

Sixty flasks containing the sterilised medium were inoculated as usual and the flasks were incubated on a shaker after addition of

0.3 ml of α -pinene. Further amounts of α -pinene (0.3 ml) were added every 24 hr. Twenty flasks were removed at the intervals of 24 hr, the contents were pooled and the broth containing cells was extracted and separated into acidic and neutral fractions as described earlier. The neutral fractions were subjected to chromatographic separation on a short column (10 times weight of material) of neutral alumina (Grade I) and eluted with petroleum ether and ether. The petroleum ether fraction eluted mostly unreacted pinene and a small amount of bacterial fats. The polar fraction from the ether eluate was concentrated and weighed. This was taken as the neutral products.

The acids were recovered by acidification and extraction with ether. The acids were weighed after removal of the solvent. The details of the separation procedure are described in Chapter II. The results of this experiment are tabulated in Table 4.

TABLE 4
The effect of time on the accumulation
of metabolites

Time hr	Total products g	Neutral fraction g	Acidic fraction g
24	0.59	0.26	0.23
48	0.91	0.37	0.29
72	1.26	0.53	0.49

It was observed that if the higher initial level of α -pinene (1%) was employed and the fermentation was stopped between 36-48 hr (before all the pinene is exhausted) the yield of neutral fractions and polar acids were lower but the amount of dicarboxylic

acid (53) and monocarboxylic acids showed increases.

Large scale fermentation

In a typical fermentation 40 flasks each with 100 ml of mineral salt medium were inoculated with 2% inoculum from a 24-hr old cultures. α -Pinene (0.3 ml) was added to each flask which was incubated on a rotary shaker for 72 hr at $28^{\circ} \pm 2^{\circ}\text{C}$. Further amounts of α -pinene (0.3 and 0.4 ml) were added at intervals of 24 hr and 48 hr respectively. At the end of the incubation period (total 72 hr) the contents of the flasks were pooled and extracted once with ether. The broth was acidified and extracted with ether again. All the ether extracts were pooled, washed, dried and concentrated. The metabolites were separated into acidic and neutral fractions by washing with 5% aqueous sodium carbonate.

For actual isolation of metabolites the contents from five batches of 40 flasks were pooled together and processed. The total ether extract on concentration afforded 25 g of products. This on separation into neutral and acidic products gave ^aneutral (N_1) fraction (14.5 g) and ^{an}acidic (A_1) fraction (6.34 g). The neutral fraction on separation of polar and nonpolar fractions by 4 transfer modified Craig separation gave nonpolar (N_1 -A) fraction (8-9 g) and a polar (N_1 -B) fraction (4.5 g).

The butanol extract was concentrated on a water bath under suction. The residue was taken up in chloroform and separated into neutral (N_2) fraction (2.1 g) and acidic (A_2) fractions (0.25 g).

The broth after extraction with ether and butanol was neutralised with ammonium hydroxide, concentrated to a small volume (200 ml) and extracted continuously with ether before and after

acidification. The continuous ether extract afforded a neutral gummy material (300mg) fraction (N_3) and a viscous acidic fraction (A_3) (400 mg).

Chromatography of the neutral fraction:

In the initial experiments the neutral fraction as such was chromatographed over alumina. In later experiments the neutral fraction was separated into polar and nonpolar and worked separately.

In a typical experiment the neutral fraction (12 g) was loaded onto an alumina column (Grade I) 150 g and the following fractions were collected.

- N_1 -a petroleum ether
- N_1 -b ether and petroleum ether (1:3)
- N_1 -c ether and petroleum ether (1:1 - 3:1)
- N_1 -d ether fraction
- N_1 -e methanol fraction

The petroleum ether fraction (N_1 -a) (7 g) was distilled b.p. $55^\circ/10$ mm (bath temp.), α_D^{21} . This material was found to be identical with α -pinene. The residual material remaining in the flask was of fatty origin.

From the tail fractions of petroleum ether eluates a small amount (20 mg) of a liquid was isolated. IR ν max 2960, 1443, 1370, 1368, 1087, 886 and 817 cm^{-1} . Found C, 80.2, H, 10.8%. $\text{C}_{10}\text{H}_{16}\text{O}$ requires C, 78.89, H, 10.59%. The VPC of the compound indicated that it contained 3 peaks (Column P, temp. 115° , H_2 gas, 50 ml/min, retention time 0.4, 0.8 and 1.6 min).

The next fraction (N_1 -b) gave small amounts of a material (70 mg)

which exhibited two spots (IR ν max 1750, 1720, 1695, 1345, 1055, 1028, 955, 823 and 753 cm^{-1}). Found C, 77.0, H, 9.8%. Both the compounds moved in TLC very close to each other and it was not possible to separate them further.

The major component from the mixture was obtained in fraction N₁-c and was a solid (2.13 g). TLC indicated that this fraction contained a major component with small amounts of impurities. Further purification was achieved by sublimation (110°C/711 mm). The sublimate appeared as shining flakes, m.p. 203°-205° (sealed capillary (lit. d borneol m.p. 203°), $[\alpha]_D^{+37}$). Found C, 77.27, H, 11.6%, $\text{C}_{10}\text{H}_{18}\text{O}$ requires C, 77.86, H, 11.76%, IR ν max 3338, 1390, 1377, 1355 cm^{-1} . (Fig. IX); NMR (Fig.X).

The compound was identified as borneol. Mixed m.p. with an authentic sample was undepressed (203°-205°C). IR spectra of an authentic sample and microbial product were identical (Fig.IX). Acid phthalate m.p. 162-163° (lit.164.5°, Beilstein, 9, 800).

Chromic acid oxidation of the above alcohol: This was done according to the method of Poes et al (1953). The resulting compound was purified by sublimation (100°C/712 mm). The sublimate showed a m.p. 176° (lit. camphor 178°C). The m.p. was undepressed on mixing the sample with camphor (176°-177°C) IR spectrum(not shown) had bands identical with ^{that of} camphor (ν max 1750, 1445, 1385, 1375, 1041, 1015, 750 cm^{-1}).

Separation of 100% ether fraction (N₁-d):

The liquid fraction eluted with 75-100% ether in petroleum ether (0.756 g) was distilled b.p. 125°-130°C/4 mm (bath temp.) to give (0.62 g) material which showed three components on TLC. The

fraction moving in the middle had the same mobility as myrtenol. These two spots moved together in a number of solvent systems of different polarity.

The VPC of this fraction showed three peaks a, b and c with retention time of 4.3, 5.2 and 6.5 min respectively (Fig.XI) (Temp. 160°, 100:2 azelaic acid polyester column, carrier gas H₂, flow rate 9 sec/10 ml). The peak (c) corresponded to myrtenol. Mixing of authentic sample with the 100% ether fraction and VPC resulted in enhancement of peak intensity of (c) as compared to the peaks a and b (Fig.XI). By a similar technique it was possible to demonstrate that peak (b) represented borneol. It was not possible to separate these compounds for further chemical studies.

This fraction (300 mg) was dissolved in 0.5 ml of dry pyridine and acetic anhydride (0.5 ml) was added dropwise. The mixture was allowed to stand overnight. At the end of the reaction the mixture was poured into water and the products were taken in ether, freed of pyridine and acetic acid by washing with dilute hydrochloric acid and water. The ether layer was dried and concentrated. The comparative TLC of the products and starting material demonstrated that all the alcohols were converted into acetates indicating that all the three compounds have a primary or secondary hydroxyl group.

The fraction N₁-e (obtained after elution of the column with methanol) was ^adark coloured gum which could not be further separated.

Acidic fraction (A₁):

Chromatography of acidic fraction on silicic acid: Silica gel (150 g) was triturated with 15 ml methanol (Arya, 1962), slurried with petroleum ether and packed into a column taking care to exclude air bubbles. The acidic fraction (5 g) was loaded onto the column with benzene:petroleum ether mixture (1:9) and fractions were eluted with solvent mixtures of increasing polarity. The fractions composed of petroleum ether, benzene and ether. TLC of the fractions collected indicated that all of them were impure. However, the benzene:ether (1:3) fraction (0.431 g) on standing in the cold deposited a few crystals of a solid (44 mg) acid (50) which on crystallisation from water melted at 155° - 156° C, $[\alpha]_D +46.7$. Found C, 65.19, H, 8.56%. $C_{10}H_{16}O_3$ requires, C, 65.19, H, 8.73. UV max $217_{m\mu}$ ϵ max 9653. IR ν max 3290 cm^{-1} (OH) (Fig.XII), 1678, 1647 (conjugated carbonyl), 2550 cm^{-1} (COOH).

Methyl ester:

The acid (50) was esterified with diazomethane in ether to give a methyl ester (51) b.p. 150° - 160° /1.5 mm (bath temp.) $[\alpha]_D +41^{\circ}$. Analysis found, C, 66.4, H, 9.2%. $C_{11}H_{18}O_3$ requires C, 66.64, H, 9.15%. IR ν max at 3503, 1720, 1660, 1263, 1092, 960, 742, 710 cm^{-1} (Fig.XIV), UV λ max $218\text{ m}\mu$ ϵ max 9806. The NMR spectrum (Fig.XIII) indicated that the compound was oleuropeic acid methyl ester (51).

The IR spectrum of the original acid was found to be identical with ^{that of} an authentic sample of oleuropeic acid. No depression in m.p. was found when mixed with an authentic sample (m.p. 155° - 156° , mixed m.p. 155° - 157°).

However, it was not possible to separate any other compound by silicic acid chromatography and therefore this step was omitted in further experimentation.

Separation of insoluble cyclohexylamine salts: The total acid fraction (5.27g) was dissolved in dry acetone (20 ml) and 3 ml of freshly distilled cyclohexylamine (BDH) in acetone (10 ml) was added dropwise with stirring. The resulting mixture was warmed on a water bath for a few minutes, cooled and allowed to remain at 0° overnight when the mixture set to a solid mass. The precipitate was filtered and washed thoroughly with dry acetone to remove adhering impurities and finally dried (1.9 g), m.p. 155°-160°C (with decomposition). Found C, 65.3, H, 12.04%. (dicyclohexylamine salt- $C_{22}H_{46}O_4N_2$ requires C, 65.3, H, 11.3%). IR spectrum^m showed bands at 2700, 2240, 1640, 1546, 1465, 1397 cm^{-1} (Fig. XV).

Regeneration of dicarboxylic acid

A slurry of the cyclohexylamine salt (1.8 g) in water was acidified with 6N hydrochloric acid and the liberated acids were extracted with chloroform. The combined extracts (250 ml) were washed with a small amount of water, dried (sodium sulphate) and evaporated to give the dicarboxylic acid (53) as a gummy mass (788 mg). The total acid was esterified with diazomethane to give dicarboxylic acid methyl ester (52) which was found to be about 90% pure on TLC (solvent 15% ethyl acetate in dioxane, developing reagent 20% nitric acid in concentrated sulphuric acid, temperature 160°C).

The methyl ester was further purified by passing over an alumina Grade II column (30 times the weight of the material). The impurities were removed by eluting the column thoroughly with

petroleum ether. The pure dicarboxylic acid methyl ester was eluted from the column with petroleum ether-ether (9:1) mixture. The fractions showing a single spot on TLC were pooled together and distilled (b.p. 140° - 150° C/2 mm (bath temp.) to give 550 mg of pure material. Found C, 62.94, H, 9.9%. $C_{12}H_{22}O_4$ requires C, 62.58, H, 9.63%. $[\alpha]_D -5.25^{\circ}$. IR ν max 2945, 1740, 1440, 1200, 1170, 1020 cm^{-1} (Fig.XVI). NMR (Fig.XIX) indicated the compound to be β -isopropylpimelic acid methyl ester (52).

Regeneration of the free acid (52):

The methyl ester (500 mg) was refluxed with excess of 5% methanolic KOH on a water bath for four hours. Methanol was removed under vacuum and the residue after dilution with water was extracted with ether to remove any unreacted methyl ester. The acid liberated after acidification of the aqueous layer with hydrochloric acid was taken up in chloroform. The chloroform layer was washed, dried and concentrated to give the free acid (374 mg). Analysis (Found, C, 59.2, H, 8.8%. $C_{10}H_{18}O_4$ requires C, 59.36, H, 8.97%). IR (Fig.XVII) ν max 2670, 1722 cm^{-1} . NMR (Fig.XVIII) agreed with the structure β -isopropyl pimelic acid.

Separation of the acetone soluble cyclohexylamine salt fraction:

The mother liquors after separation of the insoluble cyclohexylamine salts were acidified and extracted with chloroform (4x100 ml). The chloroform layer was washed with dilute acid followed by water and then dried (sodium sulphate). The evaporation of the solvent gave a viscous, coloured liquid (4.3 g). The acids were converted into methyl esters (4.2 g) by treatment with diazo-methane in ether solution. The methyl esters were further separated

into polar and nonpolar fractions by partitioning between petroleum ether and 90% aqueous methanol. The petroleum ether layer after drying and evaporation gave nonpolar methyl ester (A_1 -a) fraction (1.32 g). The methanol layer was concentrated and diluted. The polar methyl esters (Fraction A_1 -b) were recovered by extraction.

Separation of non-polar methyl esters (A_1 -a-fraction)

The non-polar methyl esters were further separated by fractional distillation. The fraction b.p. 100° - 130° /1.5 mm (bath temp.) was found to be mostly monocarboxylic ester fraction (0.97 g). The higher boiling fraction b.p. $>160^{\circ}$ C/1.5 mm (bath temp.) was shown to be a fatty acid ester (0.32 g).

Purification of the fatty acid methyl ester:

The higher boiling fraction from (A_1 -a) was purified by repeated fractional distillation (470 mg). The IR spectrum (Fig.XX) of this compound resembled closely that of the dicarboxylic acid methyl ester (Fig.XVI) but the two compounds could be differentiated by TLC. This compound was further purified by chromatography over an alumina column. The fatty acid methyl ester was freed from monocarboxylic acid methyl ester fraction by collecting the earlier petroleum ether fractions. The pure methyl ester fraction was hydrolysed and free acid was further purified by washing with cold acetonitrile (yield 212 mg), m.p. 45° C, IR (not shown) ν max 2665, 1705, 1463, 1412, 1287, 935, 719 cm^{-1} ; neutral equivalent 239; Found C, 77.39, H, 12.91% ($\text{C}_{23}\text{H}_{40}\text{O}_2$ requires C, 76.86, H, 12.90%).

The free fatty acid reacted with cyclohexylamine to give a salt which is quite soluble in acetone, m.p. 75 - 76° ; analysis (C, 73.84, H, 12.6%). IR (Fig.XXI). IR fatty acid methyl ester (Fig.XX).

Characterization of monocarboxylic acid fraction

The lower boiling fraction of methyl esters (0.96) was further purified by chromatography over alumina Grade II (30 g). The fractions eluted with petroleum ether and showing a single spot on TLC were pooled together and distilled (b.p. $110^{\circ}-135^{\circ}\text{C}/15\text{ mm}$ (bath temp.)) to give 445 mg of a material which showed a single spot on TLC. IR (not shown) λ_{max} 2940, 1735, 1713, 1433, 1254, 1198 cm^{-1} . Analysis, C, 72.04, H, 9.38%; $\text{C}_{11}\text{H}_{18}\text{O}_2$ requires C, 72.5, H, 9.96%. UV broad absorption at 220-230 m μ .

Saponification of the methyl esters:

The methyl ester (100 mg) on hydrolysis with excess methanolic KOH and working up as described earlier gave (78 mg) of a viscous liquid b.p. $140^{\circ}-150^{\circ}\text{C}/0.5\text{ mm}$ (bath temp.). Analysis C, 71.96, H, 9.78%. ($\text{C}_{10}\text{H}_{16}\text{O}_2$ requires C, 71.39, H, 9.59%). IR (not shown) λ_{max} 2670, 1700, 1436, 1300, 1296, 1270, 1218, 1148, 825, 797 cm^{-1} etc. The UV spectra had a broad λ_{max} 218-224 m μ .

The NMR spectra of both the acid and the methyl esters (not shown) presented a complicated pattern which could not be ascribed to any definite compound. The VPC examination of the methyl esters at this stage revealed that it contained seven components A to G (Fig. XXII) with retention times of 0.35, 0.51, 0.59, 0.76, 1.34, 1.65 and 1.85 min respectively (temp. 120°C , succinic polyester of diethylene glycol, carrier gas H_2 , flow rate 30 ml/min). It was not possible to separate any of the compounds in pure form by any of the physical means attempted. It was possible however to identify the methyl esters of myrtenic acid (58), phellandric acid (59) and perillic acid (39) by comparative VPC.

For further confirmation the methyl esters of authentic samples were mixed and injected. An increase in the proportion of a peak at the expense of other compounds in the mixture was taken as a criterion for the presence of the compound in the total mixture. In this manner it was possible to ascribe peak D (0.76), E (1.34) and G (1.65) to myrtenic (58), phellandric (59) and perillie (39) acid esters respectively (Fig. XXII).

Polar acid methyl esters (Fraction A₁-b):

The total ester fraction (1.95 g) was loaded on to a column of alumina Grade II (60 g). The column was eluted with the usual solvent mixtures of petroleum ether, benzene and ether and the following fractions were obtained.

- 1) A₁b-1 petroleum ether fraction
- 2) A₁b-2 petroleum ether:benzene (1:1)
- 3) A₁b-3 benzene
- 4) A₁b-4 benzene:ether (1:1)

Petroleum ether fraction (A₁b-1): The fraction of methyl esters eluted with petroleum ether (106 mg) was found to be similar to fraction (A₁a-1) corresponding to monocarboxylic acid methyl esters.

50% Benzene fraction (A₁b-2): The next fraction eluted with petroleum ether:benzene (1:1) afforded 105 mg of a liquid fraction b.p. 120°-150°/2.5 mm (bath temp.). IR (not shown) $\nu_{\max}$ 2960, 1738, 1652, 1370, 1256, 1195, 1167, 1093, 1018, 996, 898 cm^{-1} . There was complete absence of any hydroxyl band. TLC demonstrated a number of fast moving components some of which had R_f values corresponding to the esters of fraction (A₁b-1). It could

not be possible to separate this mixture into the components due to paucity of material.

Benzene fraction (A_1b-3): The earlier fractions obtained by elution of the column with benzene contained mostly nonpolar materials corresponding to fraction (A_1b-2). However the tail fraction obtained during the benzene elution was found to contain a polar hydroxylated compound. The fractions exhibiting a single spot on TLC were pooled together and distilled (26 mg, 90-95% pure by TLC). IR $\nu_{\max}$ 3500, 1732, 1258, 1200, 1167 cm^{-1} (Fig. XXIII). UV $\lambda_{\max}$ end absorption with maxima at 212 m μ , $\epsilon_{\max}$ 1013; NMR spectra (Fig. XXIV).

This compound could not be acetylated with pyridine and acetic anhydride under the usual conditions indicating the tertiary nature of the hydroxyl group.

The dehydration of this compound (5.8 mg) with pyridine (0.25 ml) and thionyl chloride (2 drops) yielded a compound (5.8 mg) which on distillation gave a material (2.8 mg). The TLC showed the absence of the characteristic hydroxy acid methyl ester spot and the appearance of a very fast moving component probably formed by dehydration of the hydroxy acid methyl ester. UV $\lambda_{\max}$ 212 m μ , $\epsilon_{\max}$ 2693.

Based on the above data the structure of the compound appears to be (61).

Ether fraction: The major fractions of the methyl esters (1.09 g) were eluted from the column with benzene:ether (1:1). Further elution of the column with 100% ether, chloroform and methanol yielded 164 mg, 35 mg and 42 mg of materials which had a number of TLC

spots in addition to two spots corresponding to the 50% ether fraction.

The 50% ether fraction showed two major components on TLC (solvent 35% ethyl acetate in Hexane). The spot moving faster of the two corresponded to the spot of hydroxy acid methyl ester (61) and was not processed further.

In order to separate the other hydroxy acid methyl ester corresponding on TLC to oleuropeic acid methyl ester, the fraction (A₂b-4), 320 mg, was rechromatographed on an alumina Grade II column (9 g). The column was washed with petroleum ether, 10 and 20% ether in petroleum ether to remove contaminating materials. The component eluted with 50% ether in benzene (238 mg) was found to be about 85% pure by TLC.

Further purification of this compound was achieved by hydrolysis with 5% methanolic KOH as described earlier. The compound recrystallized from water (103 mg) was found to be identical with oleuropeic acid (50) obtained earlier from silicic acid column. m.p. 155°-156°, $[\alpha]_D^{25} +47.5$. Found, C, 65, H, 8.59%. C₁₀H₁₈O₃ requires C, 65.2; H, 8.75%. UV λ_{max} 217 m μ , ϵ_{max} 9800. IR ν_{max} 3300, 2600, 1678, 1650, 1253, 1143, 912 cm⁻¹.

Preparation of the methyl ester: The compound (50) obtained after hydrolysis and purification (100 mg) was reesterified with diazomethane in the usual way. The methyl ester (85 mg) had IR, UV & NMR spectra identical with the ones reported earlier (Fig.XIV).

Butanol fraction (A₂): The total acids from butanol (247 mg) were esterified with diazomethane to give methyl esters (175 mg). The total esters were chromatographed over an alumina

(Grade II) column (30 times). The fraction eluted with 50% ether (95 mg) was the major component. Distillation yielded 82 mg of material b.p. 150° - 160° C/1.5 mm (bath temp.). The IR spectrum of this compound was identical with that of oleuropeic acid methyl ester (Fig. XIV).

Acids from continuous ether extract (A_3): The viscous gummy material obtained from a continuous extraction, on storage in the cold for long period, yielded a solid material (15 mg) m.p. 175° - 180° C. Found, C, 59.89, H, 8.06%. $C_{10}H_{16}O_4$ requires C 60, H, 8.0%. UV λ_{max} 218 m μ , ϵ_{max} 10737 IR (Fig. XXV) showed ν_{max} 3400, 2610, 2490, 1676, 1660, 1258, 1150, 1056 cm^{-1} . From the above data the compound appeared to be a dihydroxy acid. From the spectral behaviour the compound appeared to be identical with the compound (42) isolated by Dhavalikar (1964) by fermentation of limonene by a *Paenidomani* closely related to the present strain. However it should be mentioned that yields of this dihydroxy acid were highly variable and in many cases it could not be isolated from the continuous ether extract.

FERMENTATION OF β -PINENE

The conditions for fermentation of β -pinene were identical to those used for α -pinene. In a typical fermentation fifty flasks were inoculated as usual and incubated for 72 hr. The total fermented products obtained by ether extraction from three such batches (150 flasks) amounted to 16 gm. Separation into neutral (N_1) and acidic fractions (A_1) yielded 4.9 g of (N_1) and 5.1 g of A_1 . In the similar

way the butanol fraction yielded 2.69 g of material which was separated into neutral (N_2) and acidic fractions (A_2).

Neutral fraction (N_1): The neutral fraction on partitioning between 90% aqueous methanol and petroleum ether afforded a nonpolar fraction (N_{1a}) 3 g and a polar fraction (N_{1b}) 1.063 g.

The TLC of (N_{1a}) indicated the presence of β -pinene and small amounts of a fast moving material which was probably of fatty origin. In addition very small amounts of polar compounds corresponding to fraction (N_{1b}) were also observed.

Polar neutral fraction (N_{1b}): This fraction (0.62 g) was chromatographed over an alumina (Grade I) column (20 g). The following fractions were collected:

- 1) N_{1b-1} petroleum ether
- 2) N_{1b-2} ether:petroleum ether (1:3)
- 3) N_{1b-3} " " (1:1)
- 4) N_{1b-4} 100% ether and methanol

The fraction (N_{1b-1}) obtained by petroleum ether elution gave (75 mg) a material which corresponded to nonpolar fraction (N_{1a}).

The N_{1b-2} and earlier fractions from 50% ether eluates (N_{1b-3}) yielded (220 mg) of a compound showing a purity of 80% on TLC. This solid material was further purified by sublimation (110°C, atm. pressure). The shining white flakes (65 mg) showed m.p. 196°-200°C, $[\alpha]_D -17.9^\circ$; IR (not shown) $\nu_{\max}$ 3315, 1460, 1378, 1110, 1090, 1035, 1035, 1020, 992, 981, 937 and 832 cm^{-1} .

The IR spectrum of this compound was identical to the d(+)-borneol spectrum (Fig.IX) isolated by fermentation of α -pinene. Both the spots

exhibited some mobility on TLC plates (25% ethyl acetate in hexane).

50% Ether fraction (N_1 b-3) This fraction and the earlier fraction from (N_1 b-4) exhibited three spots on TLC. These fractions were pooled together to give (86 mg) of a material. A comparative TLC showed it to be similar to the 100% ether fraction from α -pinene. One of the spots had a R_f corresponding to borneol (47). The other may be myrtenol (49) as judged by mobility on TLC.

The 100% ether fraction and the methanol fraction gave a very small amount (30 mg) of material having a number of TLC spots which could not be further separated in quantities sufficient for chemical characterization.

The neutral fraction from butanol layer (N_2) was a highly coloured gummy material which could not be worked up further.

Acidic fraction (A_1): In the case of β -pinene fermentation the acidic fraction directly deposited a solid material on removal of solvent and cooling. These crystals were filtered and washed with ether to give a material (0.82 g) which on crystallisation from water showed m.p. 159° - 160°C $[\alpha]_D -113.9^{\circ}$. The IR spectrum of the compound (not shown) was identical with the spectrum of oleuropeic acid. The m.p. was undepressed after mixing with an authentic sample of oleuropeic acid (50).

Separation of Dicyclohexylamine salt: The mother liquors after the removal of oleuropeic acid (3.81 g) were treated with cyclohexylamine (3 ml) as described earlier using acetone as the solvent. The mixture was allowed to stand overnight and the solid filtered and washed with acetone, m.p. 150° - 155°C . The IR spectrum (not shown) of this residue was identical with that of cyclohexylamine salt of the dicarboxylic acid (Fig.XV). The free acids were regenerated from this cyclohexylamine salt (1.89 g) by acidification

and extraction and converted into methyl ester (0.82 g) b.p. 135°-140°/1.5 mm (bath temp.). The methyl esters were further purified by passing over a column as described earlier and finally distilled. In IR spectrum/^{it} was identical with the methyl ester of β -isopropyl pimelic acid (Fig. XVII) $[\alpha]_D^{25} -5^\circ$. The NMR spectrum (not shown) was identical with that of β -isopropyl pimelic acid methyl ester (Fig. XVI).

The mother liquors after separation of the cyclohexylamine salt were worked up as described earlier to get 2.5 g of unreacted acid which were converted to methyl esters (1.904 g).

The methyl esters were directly chromatographed over an alumina Grade II column (60 g) and the following fractions were collected:

- 1) A₁-a petroleum ether
- 2) A₁-b benzene
- 3) A₁-c ether
- 4) A₁-d methanol

The petroleum ether fraction (A₁-a) (0.8 g) was found to correspond to the nonpolar acids fraction of α -pinene.

The material was further separated into lower boiling monocarboxylic acid methyl esters (A₁a-1) and fatty acid methyl esters (A₁a-2) by distillation.

The higher boiling fraction of the methyl esters 150°C/1.5 mm was further purified by repeated distillation and chromatography. Found C, 76.8, H, 12.26%. C₂₁H₄₂O₂ requires C, 77.23, H, 12.96%. IR spectrum^m) max 1749, 1460, 1440, 1175, 725 cm⁻¹. The

NMR spectra (not shown) agreed with the fatty nature of this fraction.

The lower boiling fraction of the methyl esters from petroleum ether fraction (A_1 -2) corresponded to monocarboxylic acid methyl esters b.p. 85° - 130° C/1.5 mm (bath temp.). Found C, 71.94, H, 10.26%. $C_{11}H_{19}O_2$ requires C, 72.5, H, 9.38%. The IR spectra showed the absence of any hydroxyl band indicating nonpolar nature of the fraction. UV λ_{max} 220-230 m μ , ϵ_{max} 2800. The IR spectrum and VPC pattern were similar to the corresponding fraction from α -pinene. This fraction was not studied any further.

The benzene fraction (A_1 -b) (267 mg) exhibited a very complex TLC pattern in which only two clear-cut spots could be visualised. The very fast moving component corresponded to the components of fraction (A_1 -a), and the slower moving component corresponded to a hydroxyacid methyl ester spot present in the next fraction (A_1 -c) in substantial amounts.

The ether fraction (A_1 -c) (700 mg) was found to consist of mainly two spots (resolution done by TLC using 35% ethyl acetate in hexane as the solvent). One of these corresponded to the component obtained in earlier fraction (A_1 -b) and other to oleuropeic acid methyl ester.

The nature of hydroxyl group in oleuropeic acid (50) has been established earlier to be tertiary. It was, therefore, considered worthwhile to attempt the separation of these two methyl esters via preparation of acetate or acid phthalate. However none of the two compounds could be converted to these derivatives by usual methods, indicating the tertiary nature of both the compounds.

A further resolution of the two compounds was done by careful chromatography of the mixed esters (207 mg) on alumina Grade II column (20 g). The following fractions were collected:

- 1) Petroleum ether fraction
- 2) Benzene fraction
- 3) Benzene + 2% ether fraction
- 4) Benzene + 10% ether fraction
- 5) Ether fraction

The required hydroxy acid methyl ester appeared to be eluted with benzene + 2% ether fraction with a considerable amount of trailing. The fractions showing single spot were pooled together (18 mg). It showed a purity of 90-95% by TLC.

IR (in carbon tetrachloride) $\nu_{\max}$ 3400 (-OH), 1720, 1660, 1265 (α,β -conjugated ester grouping (Fig.XXVI), UV $\lambda_{\max}$ 218 m μ , $\epsilon_{\max}$ 7548).

Based on the spectral data and the resistance of the compound to acetylation only two probable structures (65) & (50) can be suggested. The NMR data (Fig.XXVII) coupled with different mobility on TLC indicated the structure to be (65).

Oleuropeic acid methyl ester:

The ether and ether:benzene (1:9) fractions afforded a methyl ester about 75-80% pure by TLC. The compound was further purified by hydrolysis and recrystallization m.p. 160°-162°. The IR spectra^m of the compound was identical with that of oleuropeic acid (50). UV $\lambda_{\max}$ 216 m μ $\epsilon_{\max}$ 10109, $[\alpha]_D^{25}$ -105°.

The corresponding methyl ester obtained by esterification exhibited the following properties. UV λ max 218 m μ , ϵ max 9865, b.p. 120°-130°/1 mm (bath temp.) $[\alpha]_D$ -99°. The IR and NMR spectra were identical with those of oleuropeic acid methyl ester (Fig. XIII & XIV).

The butanol fraction (2.69 g) on removal of solvent again yielded a solid acid (0.58 g) on cooling which was characterized as oleuropeic acid.

The mother liquors after removal of this acid were separated into neutral (N_2) 1.1 g and acid (A_2) 1 g fractions. The acidic fraction was esterified to give the methyl ester (0.780 g). TLC showed two major spots - one corresponding to hydroxy acid (B_4) methyl ester and other to oleuropeic acid methyl ester (S_1). This fraction was not processed further.



CHAPTER V

ADAPTIVE ENZYME STUDIES

ADAPTIVE ENZYME STUDIES

DISCUSSION

Even a cursory examination of the products formed during the fermentation of α and β -pinenes by the experimental strain of *Pseudomonas* (Chapter IV) indicates the existence of multiple pathways leading to the formation of different classes of oxygenated products. The formation of myrtenol and myrtenic acid indicates that the 7-methyl group of α -pinene may be progressively oxidized without the rupture of the cyclobutane ring of the α -pinene in one of these pathways. In borneol we have a case of prototropic rearrangement of the cyclobutane ring. The accumulation of perillic acid (39), oleuropeic acid (50), the hydroxy acids (61 and 65), phellandric acid (59) and β -isopropyl pimelic acid (53) on the other hand, suggests the existence of other parallel pathways which involve the complete rupture of the cyclobutane bridge. In the last mentioned compound (53) the six-numbered carbocyclic ring has also been ruptured.

In order to get further information about these pathways and the respective position of the different products in the overall scheme, it was necessary to undertake growth and adaptive enzymes studies (Stanier 1947, 1951).

Bradshaw et al (1959) reported the oxidation of camphor by a *Pseudomonas* by a reaction sequence detailed in Chapter I. The accumulation of borneol tempted one to assume in the early stages that the pathways for the pinene degradation may proceed via borneol (47) and camphor and then through a cleavage of the rings by lactonisation. However, pinene-grown cells failed to oxidise borneol (Tables 5, 6). The organism was

also found to be incapable of utilising borneol or camphor as the growth substrates. Nor were myrtenol (49), myrtenal (79) and myrtenic acid (58) and the 100% ether fraction of neutral compounds containing borneol, myrtenol and an unidentified compound oxidised by pinene-grown cells.

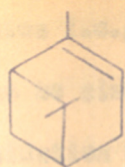
In view of the accumulation of oleuropeic acid (50), this compound and its possible precursor (α terpineol, 69) were tested with pinene-grown cells. There was little or no oxygen uptake (Tables, 5, 6). Nor do these compounds support growth of this organism.

Since borneol and oleuropeic acid accumulate in substantial amounts during the pinene fermentations, the above observations were somewhat puzzling at this stage. Unfortunately the hydroxy acid (61) and (65) was not available in quantities sufficient for growth and adaptation studies.

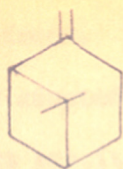
On the other hand, the total monocarboxylic acid fraction (A_1 -a) was found to be oxidised freely by the pinene-grown cells. The dicarboxylic acid was also oxidised but at much slower rates. It has been mentioned in the earlier chapter that, although it was not possible to separate the monocarboxylic acid fraction into its components, the presence of myrtenic (58), phellandric (59) and perillic acids (39) in this mixture has been demonstrated by VPC. Experiments with these compounds indicated that pinene-grown cells freely oxidised perillic and phellandric acids with equal proficiency with or without chloramphenicol but not myrtenic acid (Tables, 5, 6).

This pseudomonad is also capable of utilising limonene and Δ^1 -p-menthene as the sole sources of carbon in addition to its usual

FIG. XXVIII. SUBSTRATES USED FOR WARBURG AND ENZYMATIC STUDIES



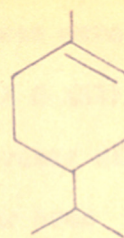
α -PINENE
(15)



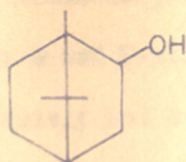
γ -PINENE
(46)



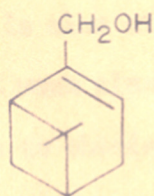
LIMONENE
(32)



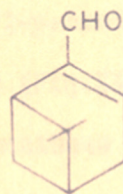
Δ^1 -p-MENTHENE
(74)



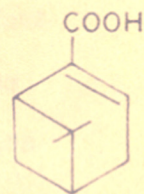
BORNEOL
(47)



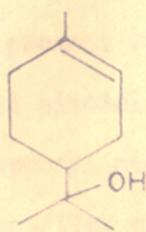
MYRTENOL
(49)



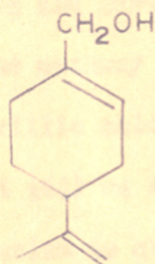
MYRTENAL
(79)



MYRTENIC ACID
(58)



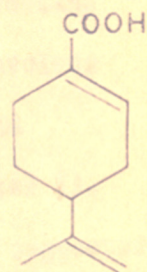
α -TERPINEOL
(69)



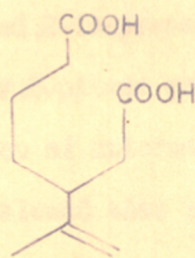
PERILLYL ALCOHOL
(43)



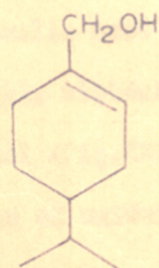
PERILLALDEHYDE
(44)



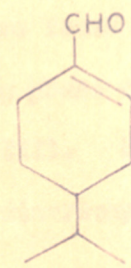
PERILLIC ACID
(39)



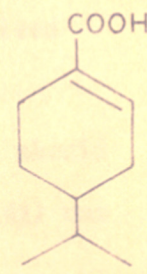
β -ISOPROPENYL
PIMELIC ACID
(41)



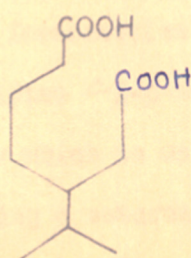
PELLANDROL
(75)



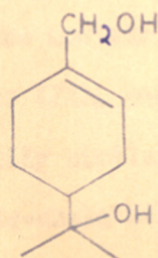
PELLANDRAL
(76)



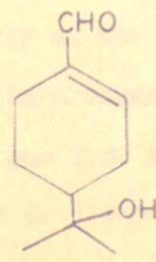
PELLANDRIC
ACID
(59)



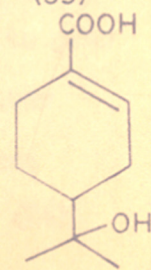
β -ISOPROPYL
PIMELIC ACID
(53)



OLEUROPEIC
ALCOHOL
(80)



OLEUROPEIC
ALDEHYDE
(81)



OLEUROPEIC
ACID
(50)

substrates i.e., α and β -pinene. The cells grown on α or β -pinene were found to be simultaneously adapted to these substrates (Fig. XXIX & XXX). This oxidation was also not sensitive to chloramphenicol. The rates of oxidation of limonene and Δ 1-p-menthene were either the same or even higher than those of the normal substrates on which the cells were grown. Limonene-grown cells also oxidise Δ 1-p-menthene at a fast rate but α and β -pinene at much slower rates (Fig. XXXI). It was unfortunately not possible to obtain Δ 1-p-menthene in quantities sufficient for further adaptation studies.

Bhattacharyya et al (1964) have observed the accumulation of a number of products during the fermentation of limonene by a variant of the present strain. The pathway for limonene degradation involves perillyl alcohol (43), perillic acid (39), hydroxy acid (40) and β -isopropenyl pimelic acid (41) as intermediates (Fig. VI, Chapter I). The capability of our organism to grow on and oxidise limonene suggested a possible similarity in the degradation pathways. The intermediates in the oxidation of limonene i.e., perillyl alcohol (43), perillic acid (39) and β -isopropenyl pimelic acid (41) were found to be oxidised by α or β -pinene grown cells without any lag phase even in the presence of chloramphenicol (Fig. XXXII, XXXIII). However, it should be mentioned that the rate of oxidation of dicarboxylic acid (41) was very slow.

The VPC evidence for the presence of phellandric acid (59) in the fermentation mixture and the capability of the organism to grow and oxidise Δ 1-p-menthene (74), suggested the presence of another pathway which is essentially similar to the one from limonene but involving a saturated side-chain at position 4. Δ 1-p-Menthene (74) phellandrol (75) and phellandric acid (59) were oxidised by α or

β -pinene grown cells without any lag phase (Fig. XXXII & XXXIII).

The saturated dicarboxylic acid - β -isopropyl pimelic acid was again oxidised at a very slow rate.

Based on the above observations it is possible to suggest probable pathways (Fig. XXXIV) for the degradation of α and β -pinene. The actual participation of the 2-hydroxy acids (40 and 77) could not be conclusively demonstrated due to non-availability of these compounds. The very slow rates of oxidation of dicarboxylic acids (41 & 53) can be explained if one assumes that they participate in these pathways in some activated form (such as CoA derivatives) and not as the free acids and the activation of the added free acids does not proceed very well in the system. In this connection it may be mentioned that Dhavalikar (1964) has presented some indirect evidence of participation of perillic acid in the activated form in limonene degradation. However the above explanations still remain to be substantiated by adequate experimental proof.

According to the overall scheme (Fig. XXXIV) borneol (47), myrtenol (49), myrtenic acid (58) and oleuropeic acid (50) are considered to be side products in the degradative pathway of pinenes.

The actual cleavage of cyclobutane ring is probably initiated by the attack of a proton on the double bond of the pinenes. The resulting carbonium ion (66) can either undergo Wagner Meerwein rearrangement to give borneol or it can rearrange to another carbonium ion (68) which is the possible precursor of all the p-menthenoid compounds obtained in these studies. It will not be out of point to mention here that this carbonium has also been implicated in the biosynthesis of α -pinene in *Pinus nigra Austriaca* by Sandermann and

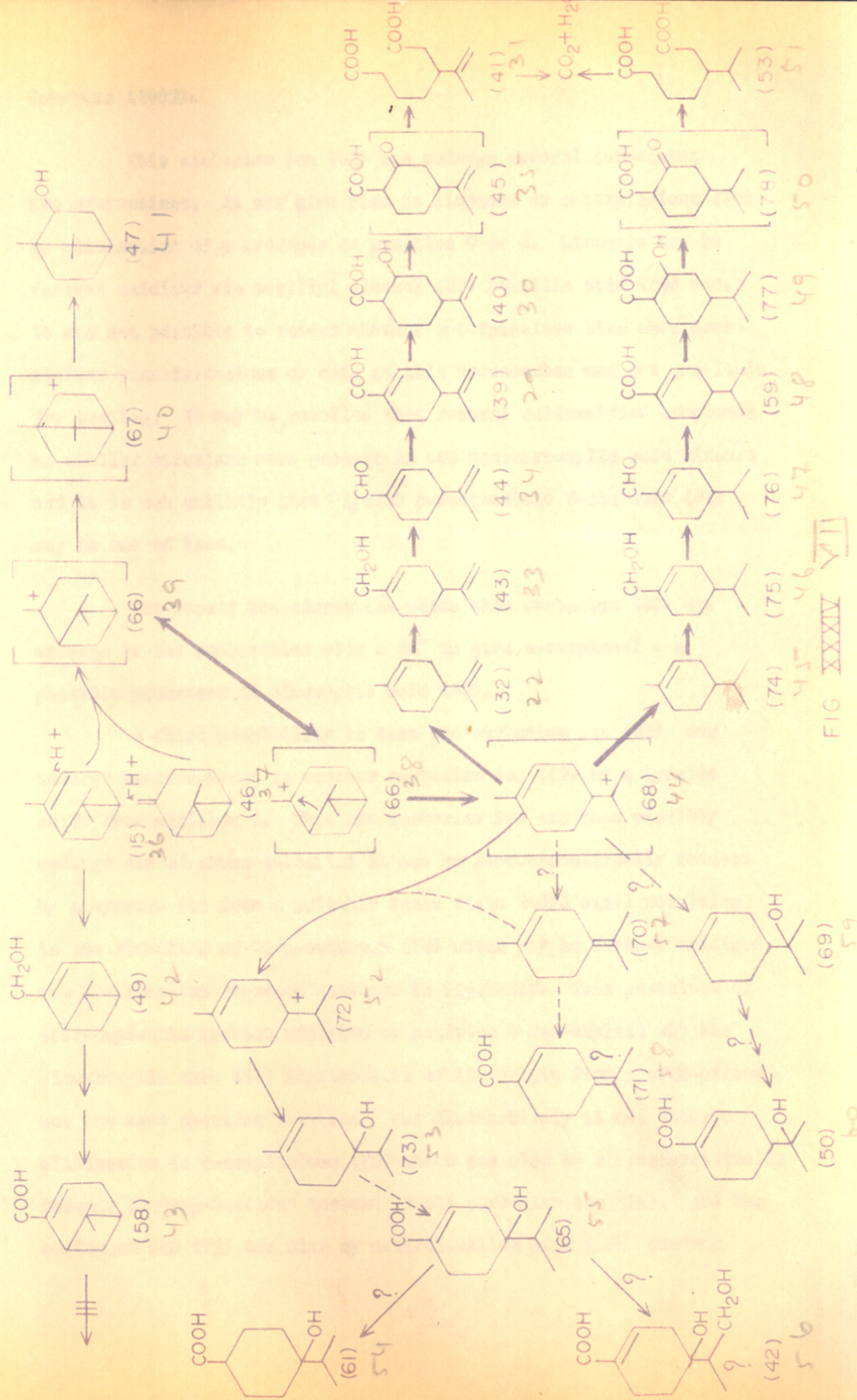


Fig. VIII PROBABLE PATHWAY(S) FOR THE DEGRADATION OF α AND β PINENES

Schweers (1962).

This carbonium ion (69) can undergo several subsequent transformations. It can give rise to limonene or α -terpinolene (70) by elimination of a hydrogen in position 9 or 4. Limonene may be further oxidised via perillyl alcohol (43) perillic acid (39) etc. It was not possible to assess whether α -terpinolene also undergoes similar transformations or not, as this hydrocarbon was not available for testing. It may be recalled that several unidentified compounds of similar structure were present in the monocarboxylic acid mixture and it is not unlikely that $\Delta^1,4(3)$ p-menthadiene 7-oic acid (71) may be one of them.

The second transformation which this carbonium ion (69) can undergo is the combination with a OH^- to give α -terpineol - a possible precursor of oleuropeic acid (50).

A third possibility is that the carbonium ion (69) can undergo rearrangement to another carbonium ion (72) by a hydride shift from position 4. This new carbonium ion can then possibly undergo one of three fates: (a) It can be stereospecifically reduced by a hydride ion from a suitable donor (like NADPH etc.) resulting in the formation of Δ^1 -p-menthene (74) which may be further oxidised via the reaction sequence outlined in Fig. XXXIV. This postulate of stereospecific hydride addition at position 4 may explain why the dicarboxylic acid (53) irrespective of its origin from α or β -pinene has the same specific rotation. (b) Alternatively it can undergo elimination to α -terpinolene (70) which can also be stereospecifically reduced to Δ^1 -p-menthene instead of the carbonium ion (72). (c) The carbonium ion (72) can also by neutralisation with a OH^- produce

4-terpineol which may be the precursor of hydroxy acids (61) and (65).

Enzymatic evidences

Attempts to demonstrate the enzyme responsible for cyclobutane ring cleavage:

The first step in the degradation of pinenes according to above scheme is a protonation followed by rearrangement, elimination and/or hydration. In this connection it is noteworthy that the transformation products obtained by simple acid catalysed rearrangement of α -pinene are borneol, limonene, α -terpineol etc. in analogous chemical reactions. Depending upon the solvents used, the products formed possess either the borneol nucleus or p-menthenoid structure (Kergomard 1956). From theoretical considerations this step does not require any oxidation (in contrast with the second step of the reaction sequence, the hydroxylating enzyme which would need one mole of O_2 per mole of substrate). It appeared, therefore, logical that the incubation of α -pinene with pinene-grown cells under nitrogen should result in the accumulation of limonene, borneol α -terpineol or Δ 1-p-menthene.

It was, however, found (Table 7) that no accumulation of a significant amount of a polar compound could be demonstrated. TLC examination of the polar fraction did not show the formation of any alcohol corresponding to borneol or α -terpineol. VPC examination of the hydrocarbon fractions (Temp. 90° , gas H_2 flow rate 70 ml/min, column P) also failed to demonstrate the presence of any component (limonene, Δ 1-p-menthene) other than α -pinene. The same experiment was repeated with the cell-free sonicates from α -pinene-grown cells to ascertain whether the failure of the reaction could be ascribed to a

permeability barrier in an active transport process which requires the expenditure of energy. Here also no reaction could be observed. Furthermore, in view of the highly non-polar nature of the substrate molecule an active transport mechanism is difficult to visualise.

In Warburg experiments the presence of 10^{-4} molar dinitrophenol did not have any significant effect on the respiration of the cells grown on α -pinene, again indicating that perhaps ATP was not involved in the actual transport of this substrate inside the cell. Higher concentrations of DNP (i.e., 10^{-3} M), however had a definite inhibitory effect presumably due to toxicity.

The failure of the protonation reaction in cell-free extracts may be ascribed to the unstable nature of this enzyme. It may be recalled that frozen pinene-grown cells lose their capacity to oxidise α -pinene. However, it is difficult to see why fresh intact cells did not carry out the protonation under anaerobic conditions. A teleological explanation will be that normally the equilibrium of the protonation may lie far towards the unprotonated hydrocarbon and the carbonium ion rearrangements from (66) to (68) and from (68) to (72) as well as the subsequent reduction by hydride (NADPH?) may take place on the enzyme surface. In this process some amounts of (67), (68) and (72) somehow leak out from the enzyme surface and give rise ultimately to borneol (47), perillid acid (39), oleuropeic acid (50), and the hydroxy acids (61 and 65) as products. If this be so, then the phellandrol pathway involving the saturated side chains may comprise the main pathway in the degradation of the pinenes. A hydride donor such as NADPH may be in short supply under totally anaerobic conditions. However, more work remains to be done before

this explanation can be accepted.

Alcohol dehydrogenase:

In order to gather evidence for these postulated pathways and to assess their relative importance as far as pinene metabolism is concerned, it was considered necessary to undertake substrate specificity studies with the enzymes involved in the steps subsequent to that of protonation. Unfortunately the hydroxylation reaction, the second step in the sequence, could not be studied as most of the cell-free preparations had very little of this activity. Therefore, these studies were restricted to alcohol dehydrogenase and aldehyde dehydrogenase the third and fourth steps in the scheme. It will be clear from Fig. XXXIVa that cell-free extracts exhibited virtually the same rates of reduction of NAD with perillie alcohol, cumic alcohol, and phellandrol. This preparation oxidised oleuropeic alcohol at a slightly higher rate. Myrtenol, however, was not oxidized under the experimental conditions.

The observed rate profile was similar to that of the purified alcohol dehydrogenase from limonene-grown cells (Ballal & Bhattacharyya, 1965). When the substrates were mixed the rates of NAD reduction were not additive, indicating the involvement of the same enzymatic site.

Aldehyde dehydrogenase

The aldehyde dehydrogenase in the cell-free extract also exhibited similar rates with perillaldehyde (44), phellandral (76) and surprisingly enough, myrtenal (79). Oleuropeic aldehyde (81) was again oxidised at a much faster rate (Table 9).

It is probable that the conversion of myrtenol to myrtenal may require a separate enzyme requiring different cofactors.

These studies although indicating the existence of parallel pathways in the organism for the oxidation of perillyl alcohol (43), phellandrol (73) and oleuropeic alcohol (80) to the corresponding acids do not unfortunately throw any further light about the major pathway. Respiration studies reported in Tables, 5, 6 and Fig. XXXII, XXXIII indicate that whereas perillic acid and phellandric acid are further metabolised, oleuropeic acid and myrtenic acid are side products - perhaps produced by a detoxication mechanism.

CHAPTER V

EXPERIMENTAL

Preparation of cells for manometry:

Erlenmeyer flasks (500 ml) containing mineral salt medium(100 ml) of the composition described earlier were inoculated from 24 hr growth of the organism in respective substrates. The flasks were incubated at $23^{\circ}\pm 2^{\circ}$ on a rotary shaker for 24 hr after addition of 0.3 - 0.5 ml of substrate. At the end of 20-24 hr the cells were harvested by centrifugation at 5°C . The cells were washed twice with M/20 phosphate buffer (pH 7.0) and finally suspended in phosphate buffer (M/20, pH 7.) to give a reading of 240-260 in a Klett-Summerson colorimeter at 660 m μ (1.3-1.5 mg dry wt/ml). This cell suspension was used as such for manometric studies.

Dry weight of washed cell suspensions:

Turbidity measurements were carried out using Klett-Summerson colorimeter. Dry weights were calculated from a calibration curve relating the turbidity reading of the washed cell suspension to known dry weight content of the organism.

Manometric methods:

Oxidation of the various substrates was followed by usual manometric procedures using air as the gas phase (Umbreit et al, 1959). Cell suspension containing resting cells (1.5 ml of cell suspension, R=240-260 at 660 m μ , about 1.3-1.5 mg dry wt) and 1 ml of phosphate buffer (M/20, pH 7.0) were placed in the main compartment of the flask.

TABLE 5

Oxygen uptake with neutral and acidic transformation
products of pinene, and other probable intermediates
by α -pinene grown cells

Substrates	QO ₂ ul/hr	
	without CMP	with CMP
α -Pinene	166	-
β -Pinene	150	-
Limonene	146	152
Δ 1-p-Menthene	165	171
Borneol	0.7	-
Myrtenol	1.5	-
Myrtenic acid	3	-
100% Ether fraction	3.5	-
α -Terpineol	0.5	-
Total acidic fraction	81	-
Monocarboxylic acid fraction	100	-
Oleuropeic acid	3	-
Nopinic acid	1.5	-
Perillyl alcohol	113	105
Perillaldehyde	106	-
(+) Perillic acid	107	112
(-) Perillic acid	100	-
β -Isopropenyl pimelic acid	10	-
Phellandrol	116	121
Phellandric acid	117	114
β -Isopropyl pimelic acid	6	-

CMP = Chloramphenicol

TABLE 6

Oxygen uptake with a number of probable
intermediates by β -pinene grown cells

Substrate	QO ₂ ul/hr	
	without CMP	with CMP
β -Pinene	155	-
α -Pinene	165	-
Limonene	192	188
Δ 1-p-Menthene	186	192
Borneol	0.5	-
Myrtenol	1	-
α -Terpineol	0	-
Oleuropeic acid	1.5	-
Nopinic acid	1.2	-
Perillyl alcohol	104	99
Perillic acid	128	126
β -Isopropenyl pimelic acid	11	-
Phellandrol	107	110
Phellandric acid	115	107
β -Isopropyl pimelic acid	3.5	-

CMP = Chloramphenicol

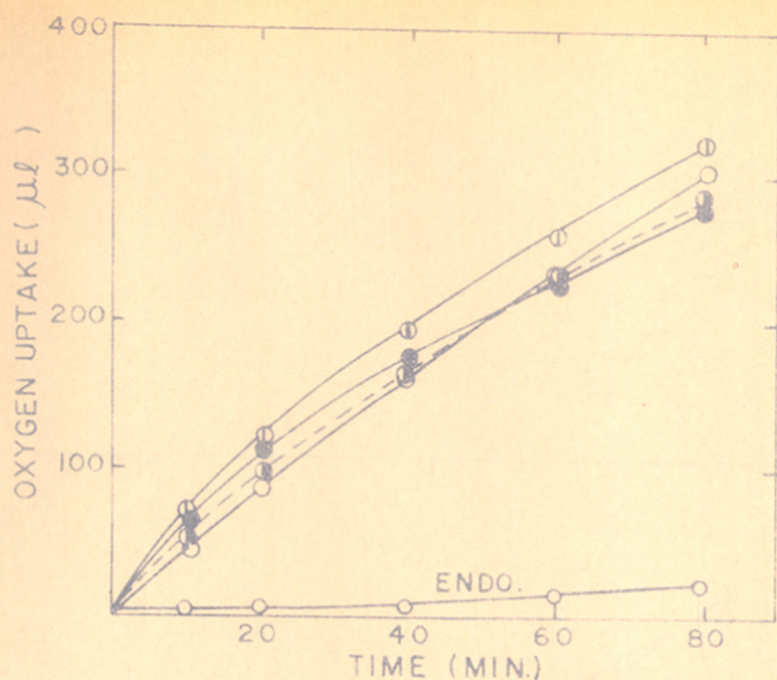


FIG. XXIX OXIDATION OF α -PINENE ($\circ-\circ$) β -PINENE ($\circ-\circ$)
 LIMONENE ($\bullet-\bullet$) AND Δ_1 -p-MENTHENE ($\bullet-\cdots-\bullet$)
 BY α -PINENE-GROWN CELLS (1.45 mg. DRY WT. PER FLASK)

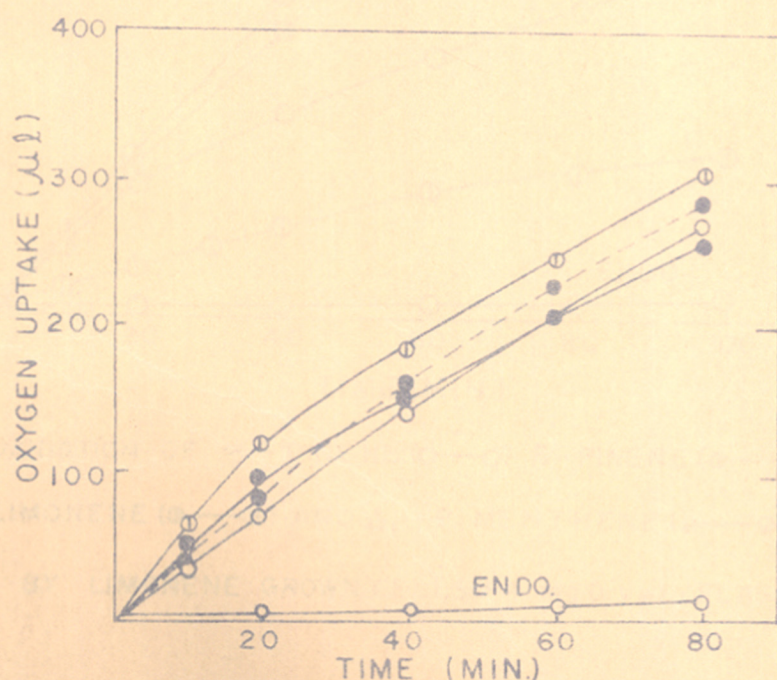


FIG. XXX OXIDATION OF α -PINENE ($\circ-\circ$) β -PINENE ($\bullet-\bullet$)
 LIMONENE ($\circ-\circ$) AND Δ_1 -p-MENTHENE ($\bullet-\cdots-\bullet$)
 BY β -PINENE GROWN CELLS (1.24 mg. DRY WT. / FLASK)

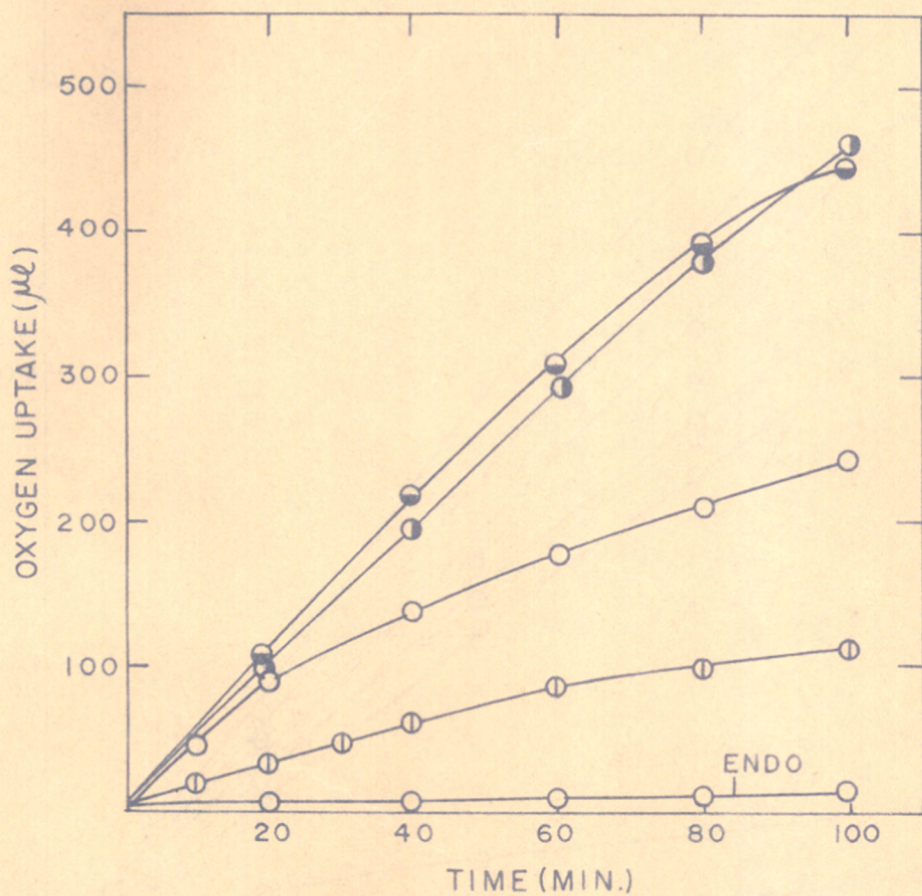


FIG. XXXI OXIDATION OF α -PINENE ($\circ$ — $\circ$) β -PINENE ($\odot$ — $\odot$)
 LIMONENE ($\bullet$ — $\bullet$) AND Δ^1 -P MENTHENE ($\bullet$ — $\bullet$)
 BY LIMONENE GROWN CELLS (1.41 mg. Dry wt./FLASK)

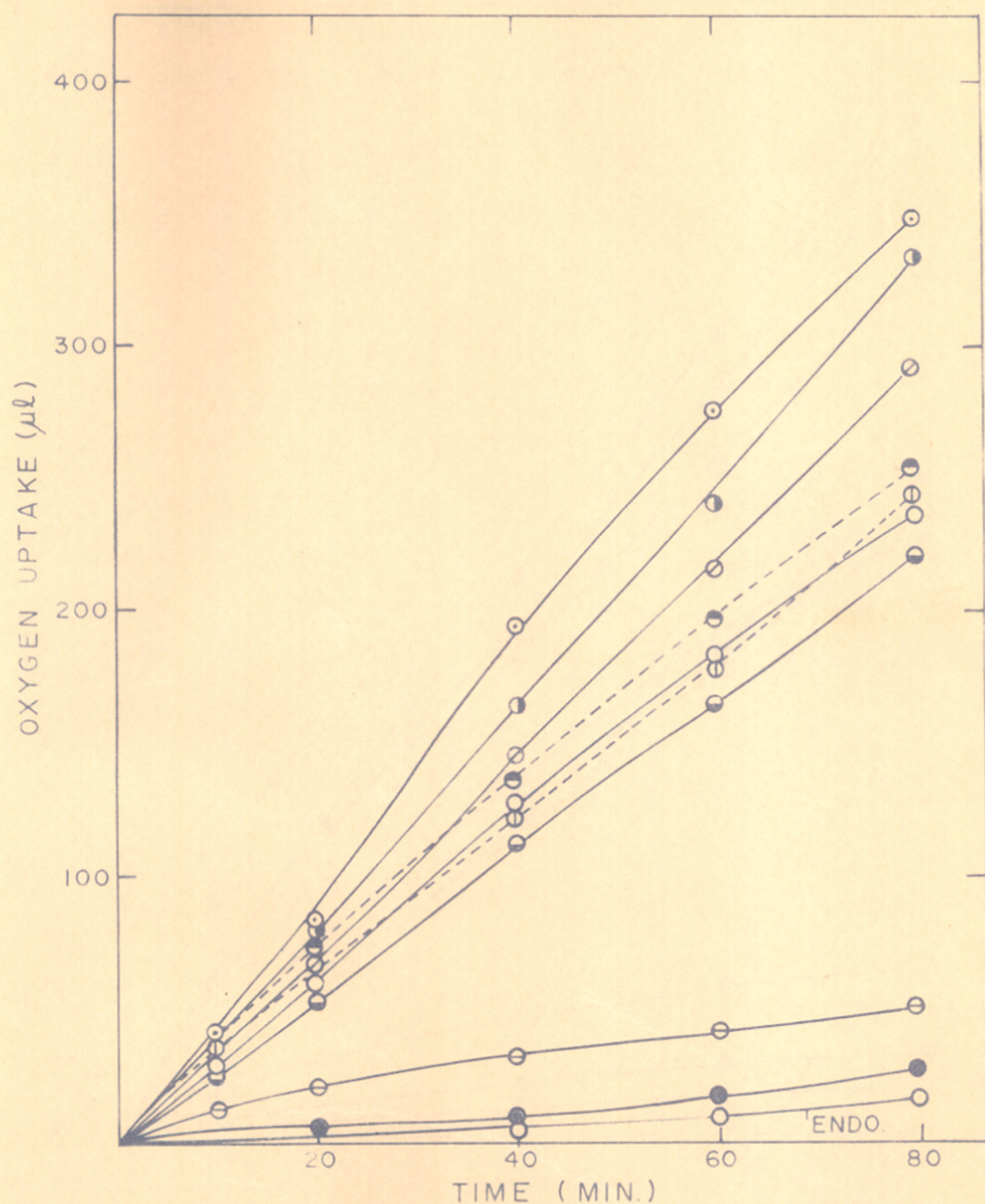


FIG. : XXXII OXIDATION OF VARIOUS SUBSTRATES BY
 α -PINENE GROWN CELLS
 (1.44 mg. Dry wt./FLASK)

○—○ α -PINENE, ○----○ LIMONENE, ●—● Δ^1 P-MENTHENE
 ○—○ PERILLYL ALCOHOL, ○—○ PERILLIC ACID
 ●----● PHELLANDROL, ○----○ PHELLANDRIC ACID
 ○—○ β -ISOPROPENYL PIMELIC ACID
 ●—● β -ISOPROPYL PIMELIC ACID

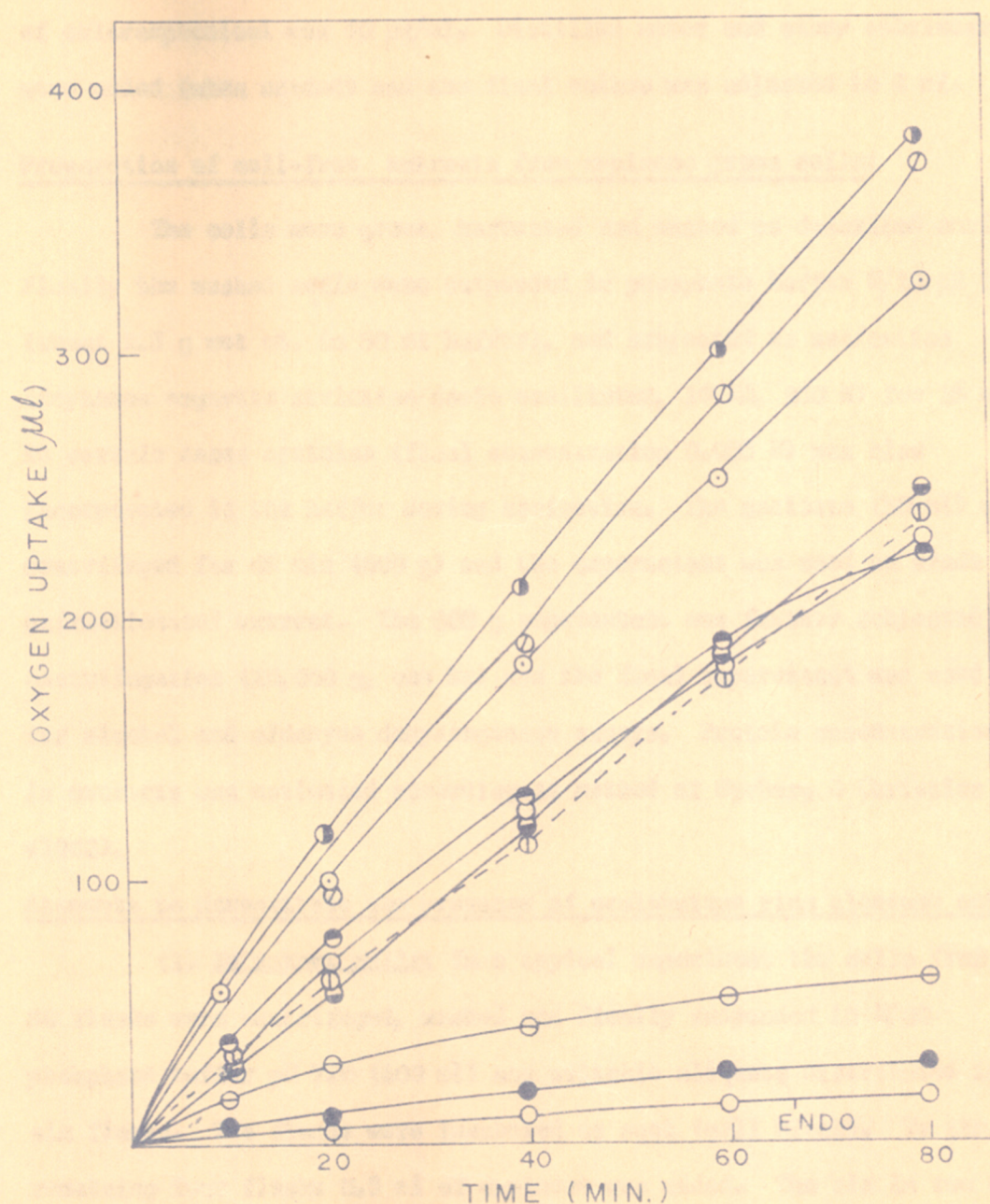


FIG. XXXIII OXIDATION OF VARIOUS SUBSTRATES BY
 β -PINENE GROWN CELLS
 (1.35 mg. Dry wt./FLASK)

- $\circ$ — $\circ$ β -PINENE, $\odot$ — $\odot$ LIMONENE, $\circ$ — Δ Δ IP-MENTHENE
 $\circ$ — $\circ$ PERILLYL ALCOHOL, $\bullet$ — $\bullet$ PERILLIC ACID
 $\bullet$ — $\bullet$ PHELLANDROL, $\oplus$ — $\oplus$ PHELLANDRIC ACID
 $\circ$ — $\circ$ β -ISOPROPENYL PIMELIC ACID
 $\bullet$ — $\bullet$ β -ISOPROPYL PIMELIC ACID

The substrates (10-20 μ M) were placed in the side arm. Central well contained 0.2 ml of 20% (w/v) KOH. When used the final concentration of chloramphenicol was 40 μ g/ml. Distilled water and other substances were added (when needed) and the final volume was adjusted to 3 ml.

Preparation of cell-free extracts from α -pinene grown cells:

The cells were grown, harvested and washed as described earlier. Finally the washed cells were suspended in phosphate buffer M/20, pH 7.0 (about 2.5 g wet wt. in 30 ml buffer), and subjected to sonication (Raytheon magnetic striction sonic oscillator, 10 KC, 250 W) for 15 min. In certain cases cysteine (final concentration 0.005 M) was also incorporated in the buffer during sonication. The sonicate (30 ml) was centrifuged for 45 min (800 g) and the supernatant was used as crude unfractionated extract. The 800 g supernatant was further subjected to centrifugation (20,000 g, one hr) and the final supernatant was used for alcohol and aldehyde dehydrogenase assays. Protein concentration in extracts was estimated according to method of Warburg & Christian (1942).

Attempts to demonstrate the presence of cyclobutane ring cleavage enzyme:

(1) In intact cells: In a typical experiment the cells from 30 flasks were centrifuged, washed and finally suspended in M/20 phosphate buffer pH 7.0 (600 ml) and suitable aliquots distributed into six flasks. Two flasks were incubated as such (cell blank). To the remaining four flasks 0.5 ml of α -pinene was added. The air in two of these flasks was replaced with nitrogen (nitrogen experimental) and other two were incubated as such (air experimental). All the flasks were incubated on a shaker at 23° for 24 hr. Two flasks containing only buffer and pinene (0.5 ml) were also processed in a similar manner (substrate blank).

TABLE 7

Product accumulation pattern, during incubation of
 α -pinene with pinene-grown cells, under aerobic & anaerobic
conditions (details see text)

Experiment	Acid fraction	Total Neutral fraction	Neutral fraction pet.ether and ether fractions (polar & non-polar)	
Substrate blank	4.5 mg	440.4 mg	PE	229.8 mg
			E	7.5 mg
Cell blank	16.6 mg	36.8 mg	PE	7 mg
			E	27.3 mg
Expt. under air	99.6 mg	126.7 mg	PE	15.7 mg
			E	23.5 mg
Expt. under N ₂	25.4 mg	788 mg	PE	562 mg
			E	38.6 mg

PE - pet. ether (non-polar fraction)

E - ether (polar) fraction

At the end of incubation period the flasks were removed, extracted after acidification and separated into acidic and neutral fractions as described earlier. The neutral fractions were separated into nonpolar and polar fractions by passing over alumina Grade I column (10 times) and eluting with petroleum ether and then ether. All the fractions were concentrated and weighed. The results are tabulated in Table 7. TLC examination of polar neutral fractions could not demonstrate the accumulation of any compound. VPC of the nonpolar fractions also failed to demonstrate the presence of any other component except α -pinene.

2) Crude extract:

In a typical experiment the sonic extract (800 g supernatant prepared from 12-15 g cells) was distributed into two flasks and α -pinene (0.5 ml) was added to each flask. The air in one of the flasks was replaced with nitrogen and both the flasks were incubated on a shaker for 24 hr. Suitable substrate and extract blanks were also incubated under identical conditions. At the end of the incubation period the contents of each flask were extracted with ether after acidification. The nonpolar and polar neutral components and acidic fractions were separated as described earlier. No accumulation of any product was observed in polar or nonpolar neutral components as judged by TLC and VPC.

Alcohol dehydrogenase

Alcohol dehydrogenase activities in 20,000 g supernatant were estimated by extent of NAD reduction in presence of suitable aliquots of enzyme (20,000 g supernatant) and substrate. The NAD reduction was followed by measuring the optical density change at 340 m μ in a

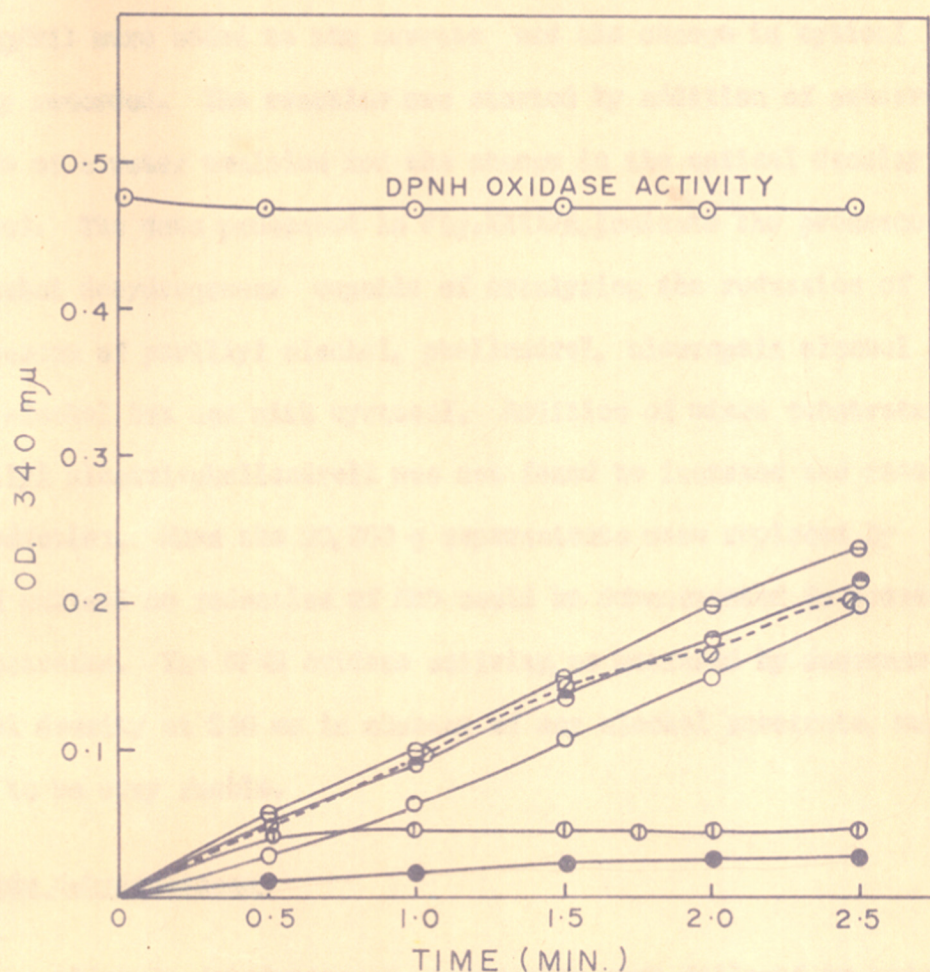


FIG. XXXIV A ALCOHOL DEHYDRGENASE

THE TEST SYSTEM CONTAINED PHOSPHATE BUFFER (0.2 ml. pH, 7.0, M/20), NAD^+ (1 mg.), substrate (1 mg. as - SUSPENSION), ENZYME (0.4 mg. - cell free supernatant) IN TOTAL VOLUME OF 3 ml.

- CONTROL (no substrate), ○—○ PERILLYL ALCOHOL,
 ○----○ CUMIC ALCOHOL, ⊖—⊖ OLEUROPEIC ALCOHOL,
 ●—● PHELLANDROL, ⊕—⊕ MYRTENOL.

Beckman model DU spectrophotometer, using a 3 ml cuvette of 1 cm light path.

Buffer (0.2 ml, pH 7.0, M/20 phosphate) water 2.5 ml and NAD solution 0.1 ml (10 mg/ml) and enzyme 0.03 ml (protein content 13.2 mg/ml) were added to the cuvette and the change in optical density recorded. The reaction was started by addition of substrate in form of a water emulsion and the change in the optical density recorded. The data presented in Fig. XXXIV A, indicate the presence of an alcohol dehydrogenase capable of catalysing the reduction of NAD in presence of perillyl alcohol, phellandrol, oleuropeic alcohol and cumic alcohol but not with myrtenol. Addition of mixed substrates (perillyl alcohol+phellandrol) was not found to increase the rate of NAD reduction. When the 20,000 g supernatants were replaced by boiled extract no reduction of NAD could be demonstrated in presence of substrates. The DPNH oxidase activity, as measured by decrease in optical density at 340 mμ in absence of any alcohol substrate, was found to be very feeble.

Aldehyde dehydrogenase

Aldehyde dehydrogenase activities were followed by reduction of methylene blue in Thunberg tubes in presence of enzyme, substrate and cofactors. The experimental conditions and the results obtained are presented in Table 8. The rapidity of the reaction was judged by the shorter time needed for complete discoloration of methylene blue.

Preparation of substrates:

Oleuropeic alcohol (30): Although Kergomard (1958) had obtained the diacetate of this alcohol as an intermediate in conversion of

TABLE 8. Aldehyde dehydrogenase activity of the extract with various substrates

	Perillaldehyde		Phellandrol		Oleuropeic aldehyde		Myrtanal	
	Enzyme Blank	Blank +Enzyme	+Enzyme +Cofactors	Blank +Enzyme +Cofactors	Blank +Enzyme +Cofactors	+Enzyme +Cofactors	Blank +Enzyme +Cofactors	+Enzyme +Cofactors
Enzyme (0.5 ml extract - 6.5 mg protein)	+	-	+	+	-	+	-	+
Buffer(phosphate, pH 7.0, M/20) 2 ml	+	+	+	+	+	+	+	+
Cofactor(FMN+FA), 0.050 mg each in 0.1 ml)	+	+	+	+	+	+	+	+
Substrate 5-10 μ M	-	+	+	+	+	+	+	+
FINAL VOLUME IN EACH CASE WAS MADE UPTO 3 ml BY ADDITION OF WATER								
Methylene blue, M/5000, 0.5 ml (side tube)	+	+	+	+	+	+	+	+
Time for color disappearance	No	No	slow* (12-15 min)	6-7 min	No	7-8 min	No	3-4 min
	No	No	6-7 min	No	7-8 min	No	3-4 min	No
								6-7 min

* In other cases also a slow but definite reduction of dye was observed in absence of added cofactors (probably due to presence of certain cofactors in the extracts.)

β -pinene epoxide to perillal alcohol, it has not been obtained in pure form.

Oleuropeic acid methyl ester (300 mg) was reduced with lithium aluminium hydride (500 mg) as described by Nystrom and Brown (1947). At the end of reaction period (about 2 hr refluxing) the mixture was worked up as usual. However the recovery of the compound from aqueous phase saturation with ammonium sulphate and chloroform extraction were needed. The chloroform layer was washed carefully with saturated brine, dried and concentrated to give (180 mg) of alcohol. This compound was freed of minor impurities by passing over an alumina column (Grade I, 20 times). The column was washed with ether. The compound was eluted with methanol. The methanol layer was concentrated and the product distilled to give a viscous gummy mass which could not be crystallised. The compound was water soluble. (IR spectra, Fig. XXIV).

Oleuropeic aldehyde:

Oleuropeic aldehyde was prepared by chromium trioxide-pyridine oxidation of the above alcohol as described by Poes et al (1953).

The methods for the preparation of other substrates will be presented in Chapter VI.

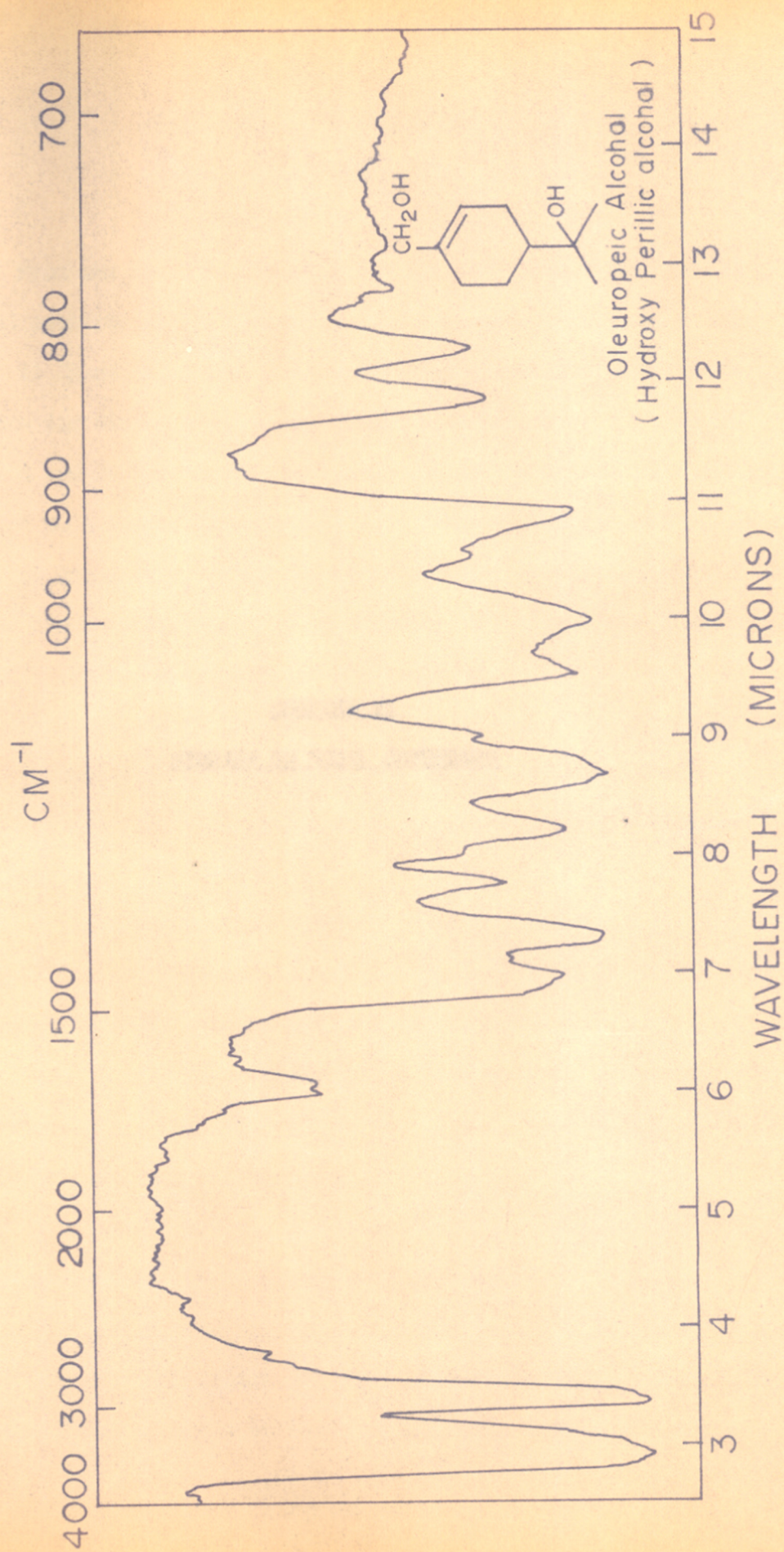


FIG. XXXV

CHAPTER VI
STUDIES ON MODEL COMPOUNDS

CHAPTER VIa
STUDIES ON MODEL COMPOUNDS
DISCUSSION

From the preceding chapter the following basic pattern of chemical activity of the bacterium on the pinenes became apparent.

1) Cleavage of the cyclobutane ring presumably through the carbonium ion (66) formed by the attack of a proton on the double bond of α and β -pinene. The rearrangement of the cation (66) may then give rise to a number of compounds as discussed in the earlier chapter.

2) Progressive oxidation of the methyl group at position 7 in the p-menthene nucleus derived by rearrangement from pinenes eventually leading to the 7-carboxymenthene derivatives.

3) Hydration of the 1-2 double bond to form the 2-hydroxy acids.

4) Oxidation of the 2-hydroxy acids to the corresponding β -substituted pimelic acid presumably through the unstable β -keto acid intermediates.

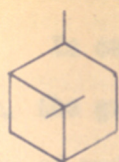
It was also observed that the organism exhibits a certain degree of versatility in utilising α -pinene, β -pinene, limonene, Δ p-menthene and p-cymene equally well as growth substrates.

From these data it appears that certain gross chemical features are essential in a molecule to make it acceptable to this *Pseudomonas* as a growth substrate. These are:

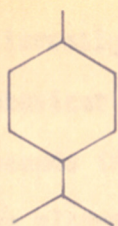
1) A p-menthane type carbon skeleton or one which can give rise to this skeleton by a prototropic rearrangement.

2) A double bond at position 1-2 or in 4-7 in β -pinene which provides the site for protonation in the rearrangement reaction and later for the addition of water to form the 2-hydroxy acids.

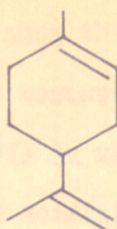
FIG. XXXVI: MODEL COMPOUNDS



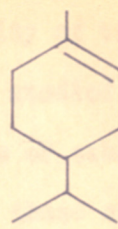
PINANE
(82)



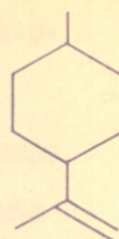
P-MENTHANE
(83)



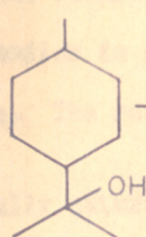
LIMONENE Δ^1
(32)



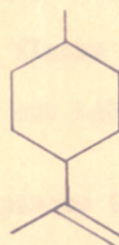
Δ^1 P-MENTHENE
(74)



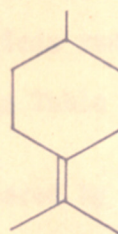
Δ^8 P-MENTHENE
(84)



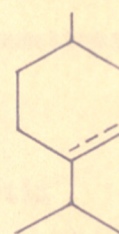
DIHYDRO-TERPINEOL
(85)



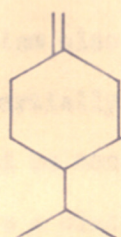
(84)



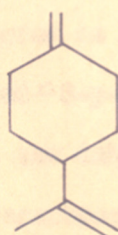
(86)



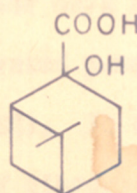
?
(87)



$\Delta^1(7)$ P-MENTHENE
(88)



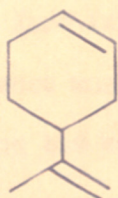
$\Delta^1(7)8$ P-MENTHADIENE
(89)



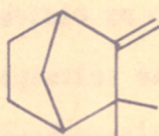
(90)



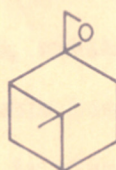
APOPINENE
(91)



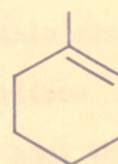
(92)



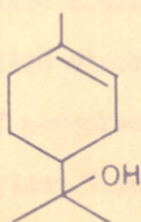
CAMPHENE
(27)



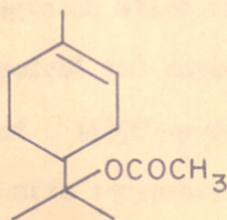
β -PINENE EPOXIDE
(93)



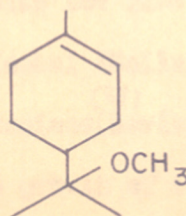
METHYL-CYCLOHEXENE
(94)



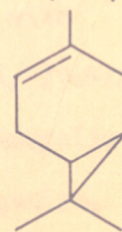
α -TERPINEOL
(69)



α -T-ACETATE
(95)



α -T-ETHER
(96)



Δ^3 -CARENE
(24)

3) A methyl/methylene group at position 7.

In order to investigate further the validity of these postulates, the growth behaviour of this bacterium was studied with a number of model compounds (Fig. XXXVI) in which one or other of these chemical features was altered. The conditions of these experiments were essentially the same as those with the normal substrates viz. the organism was incubated with these model compounds in the usual mineral salt medium in shake flasks upto periods ranging sometimes to 3 or 4 days. The results are tabulated in Table 9.

The fully saturated pinane (82) prepared by catalytic reduction of β -pinene (Lipp and Witgert 1930) lacked the 1-2 double bond which was considered to be essential for microbiological action. It did not support the growth even after four days of incubation. The organism also failed to grow on fully saturated p-menthane (83) and the partially hydrogenated Δ^8 -p-menthene (84). In this connection it should be mentioned that the dehydration of dihydro- α -terpineol (85) led to a mixture of hydrocarbons presumably (84) (86) and (87). This hydrocarbon mixture, however, showed a partial and slow response as a growth substrate. Since neither (86) nor (87) was available to us in pure form it was not possible to establish, whether or not, this growth response was due to a migration of the double bond to 1-2 position during the metabolic transformations. It is also noteworthy that the fully saturated p-menthane-7-ol is not acted upon by the purified perillie alcohol dehydrogenase which exhibits the same rates with perillie alcohol, phellandrol and cumic alcohol (Ballal et al, 1965). Δ^{1-7} p-menthene (88) and 14⁽⁸⁹⁾/8-p-menthadiene⁽⁸⁹⁾ having an exocyclic methylene like β -pinene also supported the growth of this organism.

Furthermore, nopinic acid (90) obtained through controlled

oxidation of β -pinene according to the method of DuPont (1930) did not function as a growth substrate. Anionotropic rearrangement of nopinic acid to perillic acid and oleuropeic acid has been achieved by Herz and Wahlborg (1962) and Kergomard (1957). Failure of the organism to grow on nopinic acid seems to rule out such a possibility.

The essentiality of the 7 methyl or methylene group is suggested by the fact that the organism failed to grow on apopinene (91). It was not possible to test (4-isopropenyl- Δ 1-cyclohexene (92)) as a growth substrate. However as it will be demonstrated in the following section, although the organism grows freely in p-cymene, the demethylated hydrocarbon cumene (100, Fig. XXXVII) failed to support growth. Substitution of the 7-methyl by a methoxy group or an ethyl group in p-cymene also resulted in inactive compounds.

The stereochemistry of the cyclobutane bridge in α or β -pinene or the configuration of the isopropyl or isopropenyl group in p-menthenes is of little importance as far as this organism is concerned, as it grows equally well on dl α -pinene, l- α -pinene, β -pinene, and (+) and (-) perillic acids. However after prolonged incubations on α -pinene a variant of this organism (α strain) has been isolated which apparently has lost its capacity to grow on β -pinene. Perhaps this variant may have acquired some greater degree of stereospecificity in the initial protonation step leading to the ring cleavage. β -Pinene probably does not fit in any longer on the enzymatic surface in this (α) strain.

Borneol, camphor and camphene (27) failed to serve as the carbon source for the growth of the pseudomonad although borneol has been shown to accumulate in substantial quantities during the fermentation of α -pinene. From the data on hand it can be concluded that

this compound is a side product in the ring cleavage reaction having its origin in the protonated species (66) and has no further role to play in the main pathway of pinene degradation. Borneol not only fails to support growth but in higher concentrations inhibits the growth of the organism on α -pinene. The total inability of the organism to grow on camphor or borneol distinctly differentiates it from the camphor degrading organism of Gunsalus and co-workers (Hedegaard et al, 1961).

Besides borneol, the organism also accumulates a few other compounds which appear to be side products. The presence of myrtenol and myrtenic acid in the extractives from the fermentation has been demonstrated by VPC. The question may be raised, whether or not, the cleavage of the cyclobutane ring at the stages of myrtenol, myrtenal or myrtenic acid through a protonation at the double bond may provide alternate pathways to the p-menthane type of compounds. The prohibitive amount of energy requirement to create a carbonium ion adjacent to a carbonyl function through protonation in an α - β unsaturated carbonyl system in the case of myrtenal or myrtenic acid makes alternate pathways from these compounds unlikely. Furthermore, some experimental evidence is available from which it can be deduced that the ring cleavage reaction is also unlikely at the myrtenol stage. Philibert and Kergomard (1958) failed in their attempts to prepare perillic alcohol from myrtenol by a prototropic rearrangement. This failure has been ascribed to the perturbation of the electrophilic property of the α -pinene molecule by the introduction of the hydroxyl group. In myrtenol the presence of the polar oxygen interferes drastically with the electron releasing inductive effect of the methylene group. As a result the polarity of the ring carbon 4, the

protonation site is drastically reduced. Besides oxygenation also reduces the conjugation between the ring and double bond (Philibert and Kergomard, 1958; DuPont and Dolulu, 1936). The observed failure of the organism to oxidise myrtenol, myrtenal and myrtenic acid or to utilise them for growth is in keeping with the postulate that these are side products. It is noteworthy in this connection that the bacterium did not show any difficulty in metabolising perillie alcohol and perillie acid, compounds in which the problem of opening the cyclobutane ring did not exist.

It is worthwhile to consider at this stage whether any other electrophile such as hydroxyl may also initiate the ring cleavage reaction at the pinene level through the epoxide. In fact several alcoholic compounds in the p-menthane series such as perillyl alcohol (43), phellandral (76) and hydroxyperillie alcohol diacetate (oleuropeic alcohol diacetate) have been chemically synthesized by the action of acetic anhydride and acetic acid on β -pinene epoxide (Kergomard and Philibert, 1958). β -Pinene epoxide (93) was therefore tested as a growth substrate. The organism totally failed to grow on this compound indicating that it may not be involved as an intermediate in the degradative pathway.

The presence of the isopropyl or isopropenyl side chain in the p-menthene nucleus appears to be essential for the optimal growth of the organism. Some detailed data will be presented in the following section on the effect of the nature of the alkyl group at position-4- in p-cymene nucleus on the growth of the organism. As a model compound 1-methyl- Δ -1-cyclohexene (94) failed to support the growth of the organism.

Oxygenation at position-3 in the isopropyl side chain made most of the p-menthenoid model compounds unsuitable as a growth substrate. α -Terpineol (69) not only fails to support the growth of this bacterium but is also a powerful inhibitor of its growth on limonene. Even concentrations of 0.05 to 0.1% along with 0.3% limonene are sufficient to inhibit the growth completely. Attempts to abolish the polar or hydrophilic nature of the hydroxyl group of α -terpineol by acetylation or methylation did not render the resulting acetate ester (95) or methyl ether (96) suitable as a growth substrate. However it is difficult to judge at this stage whether this inability can be ascribed to steric hindrance introduced in attempts to protect the 3-hydroxyl group. That such factors are involved may be deduced from the observation that Δ -3-carene (24) having a cyclopropane bridge connecting positions 6 and 8 is not a suitable carbon source for this bacterium.

Like α -terpineol all the other 3-hydroxy compounds such as 3-hydroxy-p-cymene (113) (Fig.XXXVII), oleuropeic acid, etc. tested so far were found to be inactive as growth substrates. The inability of the organism to grow on α -terpineol is intriguing, particularly in view of the high activity exhibited by perillic alcohol (43) and perillic aldehyde (44) dehydrogenases towards oleuropeic alcohol (90) and oleuropeic aldehyde (81). Furthermore, oleuropeic acid is one of the major products that accumulate during the growth of the organism on pinenes. It is not unlikely, therefore, that from the cation (68) some α -terpineol is produced inside the cells and is eliminated ultimately as oleuropeic acid as a detoxication mechanism. Added α -terpineol does not seem to get inside the cell.

The observed results with the model compounds thus substantially support the concepts developed in the earlier enzymatic studies (Chapter V) regarding the gross stereochemical features, essential structural elements and functional groups required in the molecule before it can serve as a sole source of carbon for this organism. However, unfortunately it is not possible to alter functional groups without introducing some changes, however, small, in the geometry of the substrate molecules. Even hydrogenation of the double bonds in pinenes and limonene also changes the geometry of the 7-methyl group drastically. In spite of these shortcomings, the results with the model compounds indicate that the organism follows its own chemical logic to degrade these terpenoid substrates. It refuses to accept as a growth substrate any compound in the degradation of which this logic cannot be applied.

CHAPTER VIIa

EXPERIMENTAL

The sources of α -pinene, β -pinene, limonene and p-cymene have already been mentioned in an earlier chapter. The samples of (+) phellandrol and Δ^1 -p-menthene were generous gifts from Dr. Persch of Drageco Laboratories, W.Germany. Δ^3 -p-Menthene, Δ^1 -7-p-menthene and Δ^1 (7)8 p-menthadiene were obtained from Dr. Klein of the same Laboratory.

The samples of (-) perillie alcohol and (-) perillie acid $[\alpha]_D^{25} -91$ were prepared from β -pinene epoxide according to the method of Kergomard (1958). The (+) perillie alcohol was prepared by lithium aluminium hydride reduction of (+) perillie acid $([\alpha]_D^{25} + 96)$ according to the method of Nystrom and Brown (1947). The samples of (+)perillie acid prepared by fermentation of (+) limonene by a soil pseudomonas in the presence of glucose were kindly supplied by Mr N. R. Ballal of this Laboratory. The samples of Δ^3 -carene were obtained by fractionation of Indian turpentine oil through a batch-stripping distillation method under total reflux.

Pinene was prepared from hydrogenation of β -pinene with platinum oxide catalyst (1-5%) at atmospheric pressure and room temperature (Lipp and Witgert, 1930). Any unreacted material was removed by repeated washings with conc. H_2SO_4 . The material had a b.p. 164.5-165.5/711 mm, $\alpha_D -8.3$. Apopinene (b.p. 138-139°/711 mm) was prepared according to method of Eschiazzi and Pines (1959) by decarbonylation of myrtenal.

Myrtenal was obtained by selenium dioxide oxidation of α -pinene (Ipatieff et al, 1951) in good yields. The corresponding alcohol and acid were prepared by lithium aluminium hydride reduction and silver oxide oxidation of the aldehyde.

p-Menthane was obtained by hydrogenation of limonene with Adams catalyst in almost quantitative yield. Any unreacted material was removed by washing with conc. H_2SO_4 .

The sample of nopinic acid was prepared by potassium permanganate oxidation of β -pinene as described by DuPont (1930). Better yields were obtained by carrying the reaction for only 30 min. The potassium salt obtained according to this method was further purified by dissolving in hot water and precipitating with excess alcohol.

The samples of borneol were obtained as a fermentation product of α -pinene as described elsewhere. The sample of camphene was obtained from L. Light & Co. and d+ camphor was obtained from Fluka.

β -Pinene epoxide was prepared by perbenzoic acid oxidation of β -pinene in chloroform (Prilejaiev et Vercheuk 1929, 1933). The perbenzoic acid was prepared as described in literature (Braun, 1946, or its modification, Kolthoff et al, 1947).

α -Terpineol was a sample of W.J. Bush and Co. Ltd. London. α -Terpinyl acetate and α -terpinyl methyl ether were prepared according to methods of Kergomard (1956) and Bayer (Ber. 24, 326, 2560) respectively. In the latter procedure metallic potassium was used in place of an alloy of sodium and potassium. The purification of the terpinyl methyl ether was achieved by passing over a neutral alumina (Gr. I) column. The petroleum ether eluates were further purified by distillation (b.p. $99-101^\circ/16-18$ mm). It exhibited a single spot on TLC. Dihydroterpineol was obtained by catalytic hydrogenation of α -terpineol. A hydrocarbon mixture was obtained from this by

dehydration with KHSO_4 . The NMR spectra indicated it to contain (34), (36) and small amounts of (37). The purity of the various samples used in these studies was ascertained by thin layer chromatography or vapour phase chromatography.

Growth studies:

All the growth studies were performed by incubating the bacteria in mineral salt medium containing the different substrates as the sole source of carbon in shake flasks. The growth was recorded either visually or turbidimetrically. The results of the visual recording of growth behaviour are presented in Table 9.

TABLE 9

Growth of the organism on various compounds

Compound	Concentration ml/100 ml	Growth 24 hr	Growth 48 hr
α -Pinene (dl)	0.3 ml	+++	+++
α -Pinene (l)	0.3 ml	+++	+++
Pinane	0.3 ml	-	-
Apopinene	0.3 ml	-	-
β -Pinene	0.3 ml	+++	+++
β -Pinene epoxide	100 mg	-	-
Myrtenol	50-100 mg	-	-
Myrtonic acid	50-100 mg	-	-
Borneol	50-200 mg	-	-
Camphene	100 mg	-	-
Camphor	100-200 mg	-	-
Nopinic acid	100 mg	-	-
Limonene	0.3 ml	+++	+++
Δ 1-p-Menthene	0.3 ml	+++	+++
Δ 3-p-Menthene	0.3 ml	-	-
p-Menthane	0.3 ml	-	-
Δ 1-(7)-p-Menthene	0.3 ml	+	+++
Δ 1-(7) 8 p-Menthadiene	0.3 ml	++	+++
Δ 3-Carene	0.1-0.3 ml	-	-
Oleuropeic acid	50-100 mg	-	-

contd

TABLE 9 (contd)

Compound	Concentration ml/100 ml	Growth 24 hr	Growth 48 hr
Perillic acid (+)	100 mg	++	++
Perillic acid (-)	100 mg	++	++
α -Terpineol	0.1-0.3 ml	-	-
α -Terpineol acetate	0.1-0.3 ml	-	-
α -Terpineol methyl ether	0.1-0.3 ml	-	-
Geraniol	0.1 ml	-	-
1-Methyl cyclohexene-1	0.1-0.3 ml	-	-

- no growth; + feeble growth; ++ moderate growth
+++ good growth

CHAPTER VIISTUDIES ON AROMATIC MODEL COMPOUNDSDISCUSSION

The capacity of the organism to grow on p-cymene has been mentioned in an earlier chapter. It has also been discussed that the organism though capable of growth on limonene and Δ -1-p-menthene does not accept 1-methyl cyclohexene-1, as a growth substrate thus indicating the necessity of a substituent in position-4. The growth on a typical aromatic compound, p-cymene (97), offered an unique opportunity of testing a large number of easily accessible model compounds as substrates and deriving more information regarding the structural features essential in a compound before it can serve as a growth substrate for this bacterium.

A large number of compounds were either prepared according to known methods or with suitable modifications of the known procedures. The structures of all such compounds are presented in Fig. XXXVII. The methods of their preparation and physico-chemical properties are described in the Experimental Section. In these experiments cells freshly grown on α -pinene were used after proper washing under aseptic conditions to remove the adhering substrates. The growth pattern was followed visually and has been presented in Table 10.

The natural choice while testing aromatic compounds falls on simple basic compounds such as benzene, toluene (98) and ethylbenzene (99). However in our studies not even a trace of growth was observed with these compounds under the period of study (10 days).



P-CYME
(97)



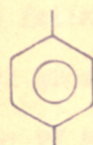
TOLUENE
(98)



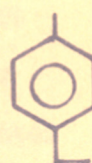
ETHYL BENZENE
(99)



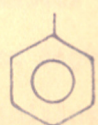
CUMENE
(100)



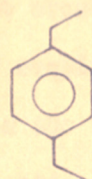
P-XYLENE
(101)



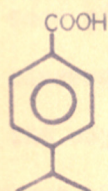
1-METHY 4-ETHYL
BENZENE (102)



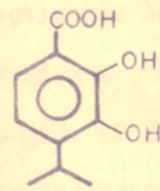
$\text{H}_3\text{C}-\text{C}=\text{O}$
p-METHYL
ACETO PHENONE
(103)



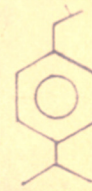
1,4-DIETHYL
BENZENE (104)



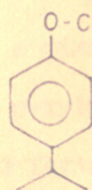
COOH
CUMIC ACID
(105)



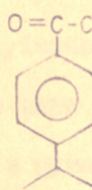
COOH
2,3 DI-HYDROXY
CUMIC ACID (106)



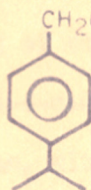
ETHYL-p-I sopr.
BENZENE (107)



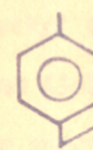
(108)



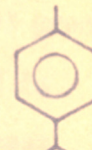
$\text{O}=\text{C}-\text{CH}_3$
p-Isopr.ACETO-
PHENONE (109)



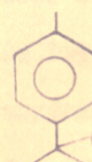
CH_2OH
CUMIC ALCOHOL
(110)



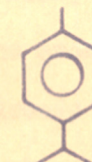
p-n-PROPYL
TOLUENE (111)



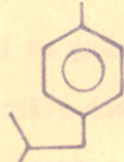
p-ISOPROPENYL
TOLUENE (112)



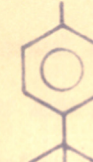
DIMETHYL,p-
TOLYL CABINOL
(113)



p-SEC-BUT
TOLUENE (114)



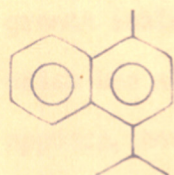
p-ISO-BUT.
TOLUENE (115)



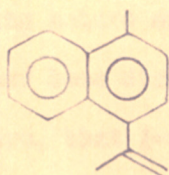
p-ter. BUTYL
TOLUENE (116)



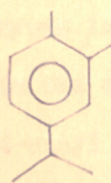
1-METHYL-4-(1-
ETHYL-PROPYL)-
BENZENE (117)



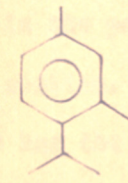
APOCADALENE
(118)



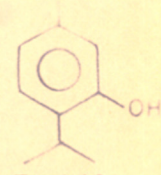
(119)



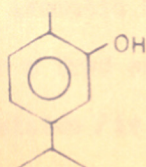
2-METHYL,p-
CYME (120)



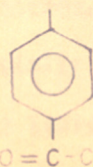
3-METHYL-p-
CYME (121)



THYMOL
(122)



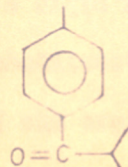
CARVACROL
(123)



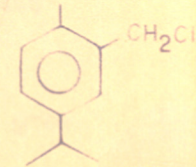
$\text{O}=\text{C}-\text{CH}_3$
p-ETHYL, ACE-
TOPHENONE
(124)



$\text{O}=\text{C}-\text{CH}_2\text{CH}_3$
p-METHYLPRO-
PIOPHENONE
(125)



$\text{O}=\text{C}-\text{CH}_2\text{CH}_3$
ISOPROPYL-p-
TOLYL KETONE
(126)



CH_2Cl
2 CHLORO METHYL
P CYME
(127)

It should be mentioned that different concentrations of these substrates were employed in an attempt to differentiate between toxicity and other effects. Among monosubstituted benzene derivatives cumene (100) which can be regarded as desmethyl-p-cymene failed to support growth indicating the essentiality of the 7-methyl group. However the possibility that the electronic effect of a o- or p-disubstituted benzene may influence the growth of the bacterium remained to be tested. It can be argued that in case of monosubstituted benzenes the higher energy requirement for cleavage of the ring may be the limiting factor in the ability of the organism to grow on such substrates. That this is probably not the case is indicated by the inability of the organism to grow on ortho xylene and para xylene (101) or a mixture of xylenes. Addition of one more methyl to the alkyl group in p-xylene as in case of 1-methyl-4-ethyl benzene (102) resulted in the formation of a growth substrate, although the adaptation process needed to grow on this substrate seems to be extremely slow, only traces of growth being observable at the end of three days. After this lag phase the growth seemed to proceed at normal rates. Further transfers from already adapted cells did not show any lag phase. In spite of the growth on this hydrocarbon the organism totally failed to adapt to p-methyl acetophenone (103) once more indicating the growth inhibiting nature of the oxygen function in the position 8 which has already been discussed in the earlier section. It appears, therefore, that 1-methyl-4-ethyl benzene has the basic minimum structural requirement for the growth of this organism on an aromatic hydrocarbon. That the organism has a rigid requirement for a free or partially oxidized methyl group substituent at position 7 is again indicated by the inability of the organism to grow

on 1,4-diethyl benzene (104).

At this stage it should be mentioned that the pathway for degradation of p-cymene follows the same pattern as with limonene in the early stages resulting in cumic acid (105) as an isolable intermediate. From the cumic acid the pathways are different. While perillic acid is degraded through 2-hydroxy-7-oic acid pathway, cumic acid is further degraded via the 2,3 dihydroxycumic acid intermediate (106) (K. M. Madhyastha & P. K. Bhattacharyya, 1965).

The essentiality of the 7-methyl group becomes apparent in the light of the above findings. It is, therefore, expected that any attempt to change the methyl group in such a way that it can no longer be easily oxidized to a carboxy group would result in an inactive compound. The failure of this organism to grow on 1,4-diethyl benzene (104) and 1-ethyl-4-isopropyl benzene (107) can be explained by this postulate. Replacement of the 7-methyl group by a methoxy group (108) or an acetyl group (109) does not convert the resulting compounds into growth substrates. On the other hand, the 7-hydroxymethyl compound (cumic alcohol (110)) itself appears to be an intermediate in degradation of p-cymene and is oxidised very fast by either p-cymene or pinene-grown cells.

Regarding the isopropyl group at position 4 in p-cymene, the organism does not seem to have an absolute steric requirement. It grows with facile ease on 1-methyl-4-n-propyl benzene also (111). The growth of the organism on 1-methyl-4-isopropenyl benzene (112) was anticipated, however, it is difficult to explain why lag phase

with this compound was prolonged upto the third day. Perhaps the organism had to come across a higher energy barrier in the form of the higher resonance energy of the extended conjugated system in this compound. As anticipated dimethyl-p-tolyl carbinol (113) having an oxygen function at the forbidden position 3 did not support growth.

Further extension of the isopropyl side chain was tolerated to a limited extent by the organism. It grows almost as freely on p-sec.butyl toluene (114) as on p-cymene, visible growth occurring after 12 hours of incubation. Surprisingly enough, p-isobutyl toluene (115) was found to be a very poor growth supporter.

p-Tertiary butyl toluene (116) presents an interesting case since it supports a very sluggish growth of the organism with the change of the coloration of the medium from colorless to yellow, then pink and finally black. The organism, however, failed to grow on 1-methyl-4-(1'-ethyl-propyl)-benzene (117) in which the isopropyl side has been extended on both the methyl groups of p-cymene.

Having assessed the stereochemical requirements of the organism at position 1 and 4 in benzene substrates, it was considered necessary to study the effect of substitution at other positions. Addition of an additional ring to p-cymene to give apocadalone (118) resulted in an inactive compound. The corresponding isopropenyl derivative, 1-methyl-4-isopropenyl naphthalene (119) also did not support the growth of this organism. In fact, the introduction of an extra methyl group in positions 2 or 3 in the p-cymene molecule as in case of 1,2 dimethyl-4-isopropyl benzene(120)

and 1,3-dimethyl-4-isopropyl benzene (121) renders the compound unacceptable to the organism as a growth substrate.

In this connection it should be mentioned that neither thymol (122) nor carvacrol (123) could support the growth of this bacterium.

The growth of the microorganisms on aromatic compounds is by no means a rare phenomenon. The utilisation of aromatic hydrocarbons for growth purposes and the mechanisms involved in the degradation of aromatic nucleus have been summarised in a number of excellent reviews (Zobell, 1946, 1950; Happold, 1950; Taussan 1950; Beerstecher 1954, Stanier 1955; Dagley et al 1960; Davis and Raymond 1961; Fuhs 1961; Regoff 1961, Evans 1963). Since this subject has been widely studied it will not be discussed here. However, the organism under study is uniquely characterised by its remarkable stereoselectivity towards the p-cymene type of aromatic substrates. It is possible that some of these compounds may act as selective agents for the screening of a particular type of cells which are normally present in a mixed population. A case in point is the development of α -strain which has lost its ability to grow on β -pinene. Another possibility is that growth on any one of these substrates may induce all the enzymes responsible for degradation of the p-menthenoid molecules. The rate pattern with perillic alcohol dehydrogenase obtained from limonene-grown cells and α -pinene-grown cells are identical with a variety of substrates. It is also not possible to rule out completely allosteric effects where the same enzyme complex is inducible through different substrates through the same operon sites.

To understand the nature of the adaptation pattern it will be necessary to work with mutants and isolated enzyme systems from cells adapted to different hydrocarbon substrates. The present studies have been restricted to elucidate the pathways for α - and β -pinene and to determine only the gross structural requirements in a substrate molecule acceptable to the organism.

Some reservations need to be made, however, before taking it for granted that the conclusions about structural specificity of a substrate molecule derived from the aromatic model systems, would also be applicable to the hydroaromatic terpenoid systems. There are some discernible differences in the biodegradation pathways of these two classes of compounds particularly in the later steps. Nevertheless, the behaviour of the organism towards the aromatic substrates appears to be dictated by if not the same, closely similar chemical logic as was evident from studies with the hydroaromatic model compounds in the earlier section.

CHAPTER VIIEXPERIMENTAL

Benzene, toluene, cumene and ethyl benzene were products of B.D.H. p-Xylene was a Fluka product and p-cymene was obtained from M/s. Riedel de Haan, Germany. All the compounds were further purified by distillation, either as such or after refluxing over metallic sodium. Substrates were sterilised by filtration. Other compounds were prepared by procedures described in literature either as such or with suitable modifications.

1a. p-Methyl acetophenone (103):

This compound was prepared by condensation of toluene and acetic anhydride in presence of anhydrous aluminium chloride (Adams and Noller, 1946, Vogel, 1956, 732). Another procedure was also employed for the preparation of this compound involving the condensation of toluene and acetic acid in presence of polyphosphoric acid (PPA) (Dev 1955, 1956). The constant boiling ketone b.p. $113^{\circ}\text{--}114^{\circ}\text{C}/12\text{--}13\text{ mm}$ gave a 2,4-dinitrophenylhydrazone, m.p. 257°C (literature 256°C , Dev, 1956).

1b. 1-Methyl-4-ethyl benzene (102):

1-Methyl-4-ethyl benzene was prepared by Clemmenson reduction of p-methyl acetophenone by the method of Brady and Day (1934). Compound (103) (8.7 gm) was refluxed with zinc amalgam (prepared from zinc wool, 20 gm mercuric chloride, 2 gm, HCl 3.2 ml and water 13 ml), 25 ml of conc. HCl and 25 ml of water for 24 hr. Small lots of conc. HCl (5 ml) were added at 6 hr intervals. At the end of this reaction period the hydrocarbon layer was separated

and the water layer was extracted with ether (4 x 75 ml). The ether extract along with the hydrocarbon layer was washed with water, dried (sodium sulphate) and distilled after removal of solvent b.p. 157°-159°/711 mm, yield 50 to 60%, VPC indicated a purity of 99%.

2a. p-Ethyl-acetophenone (124):

This ketone was prepared by condensation of ethyl benzene and acetic acid in presence of PPA as detailed by Dev (1955, 1956) for the preparation of p-methyl acetophenone. Although the PPA condensations with hydrocarbons are not very quantitative due to poor solubility of the hydrocarbon in the reaction mixture, this method could be utilised for the preparation of small amounts of this compound and a few others. All the condensations were carried out at the water bath temperatures and a period of 2 hr.

To the PPA prepared from phosphorus pentoxide (75 gm) and phosphoric acid (sp.gr. 1.84, 30 ml) was added a mixture of ethyl benzene (10.6 gm) and acetic acid (7 ml). The reactants were heated with stirring on water bath for 2 hr. The resulting mixture was poured while hot into crushed ice and allowed to stand overnight. The product was taken up in ether, washed, dried (sodium sulphate) and distilled after the removal of solvent, b.p. 122°-123°C/18-19 mm, yield 7 gm; 96-97% pure by VPC. IR spectrum ν_{max} , 1635 (C=O), 1602, 1565, 1355, 1265 and 831 cm^{-1} (para disubstituted benzene). The NMR spectrum (Fig. XXXVIII) exhibits following characteristics: a triplet, 8.77 τ , 1Hps, (methyl of $-\text{CH}_2-\text{CH}_3$), a singlet at 7.55 τ , 3 proton intensity (the methyl group of $-\overset{\text{O}}{\underset{\text{||}}{\text{C}}}-\text{CH}_3$). The two methylene protons of $-\text{CH}_2-\text{CH}_3$ appeared

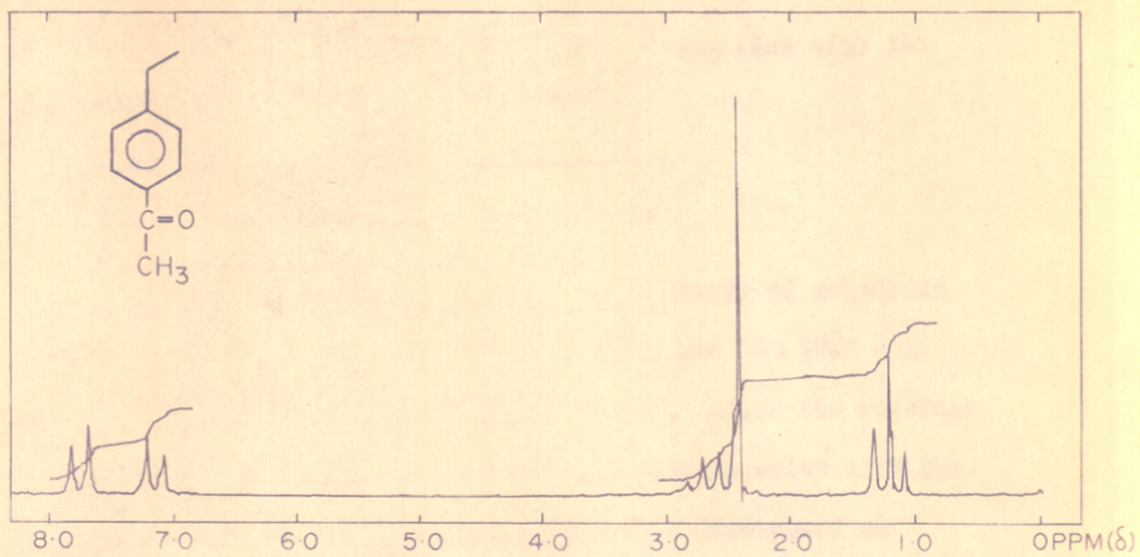


FIG. XXXVIII

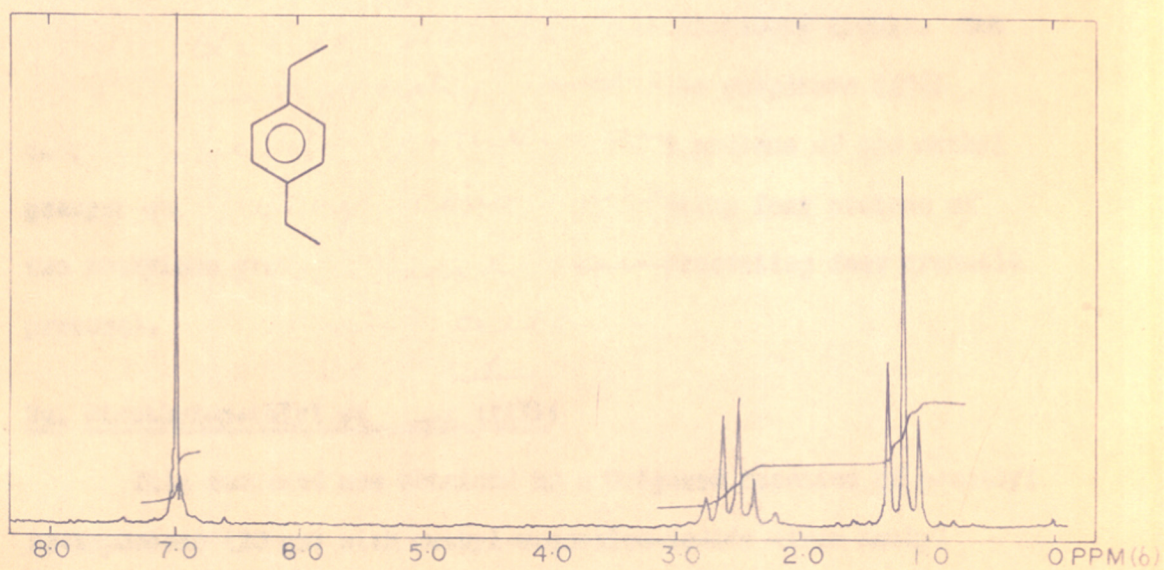


FIG. XXXIX

as a quartet (7.34 τ , J 8 cps), the fourth peak merging into the singlet of $\text{CH}_3\text{-}\overset{\text{O}}{\underset{\text{||}}{\text{C}}}$. The aromatic protons are grouped as two doublets (2.85 τ , J 8 cps and 2.25 τ , J 8 cps). The down field shift of the lower protons is ascribable to the conjugation with the carbonyl group.

2b. 1,4-Diethylbenzene (104):

Compound (124) (5 gm) was added to a slurry of anhydrous aluminium chloride (15.4 gm), ether (15 ml) and LAH (2.2 gm), prepared according to Brown and White (1957). After the vigorous reaction has subsided the mixture was refluxed on water bath for 30 min. The resulting mixture was cooled and decomposed as described by Brown and White (1957). The product was taken up in ether and washed with water. The ether layer after drying and removal of solvent gave 2.63 gm diethyl benzene b.p. 175°-180°C/711 mm. The VPC at 124°C using a polyester column indicated a purity of 98%. The IR spectrum of the compound was found to be identical with the one described in literature by Cram and Steinberg (1951). The NMR spectrum (Fig. XXXIX) corresponded with the structure (104) (a triplet at 8.8 τ , J 7.5 cps representing 6 protons of two methyl groups; quartet at 7.44 τ , J 7.5 cps representing four protons of two methylene group and a singlet at 3 τ , representing four aromatic protons).

3a. Dimethyl-p-tolyl carbinol (113):

This compound was obtained by a Grignard reaction of p-methyl acetophenone (20 gm) with methyl-magnesium-iodide (from methyl iodide (27 gm) and magnesium (5 gm)) in anhydrous ether. At the end of addition of p-methyl acetophenone to the Grignard reagent

the mixture was refluxed for 30 min and decomposed with cold dilute hydrochloric acid and the carbinol was taken up in ether. The ether layer was washed, dried and concentrated. The resulting carbinol showed a purity of 90-92% by TLC. The minor impurities were removed by passing over alumina Grade-I column (30 times). This carbinol was then used as such for growth studies.

3b. 1-Methyl,4-isoprenenyl benzene (112):

This compound was prepared by dehydration of the above carbinol (113) with potassium hydrogen sulphate. The carbinol (25 gm) was heated in an oil bath with freshly fused KHSO_4 (300 mg) at 150°C for 1 hr. The dehydration product was dried and redistilled. b.p. $180^\circ\text{--}184^\circ/715$ mm, yield 17 gm. The IR spectrum of this compound was identical with the one reported in literature by Farnow and Porsch (1964).

The NMR spectrum (Fig.XL) indicated the absence of isopropyl group, presence of two methyl signals (7.94τ - methyl group of isobutylenic side chain, 7.74τ -aromatic $-\text{C}-\text{CH}_3$) and the aromatic protons appearing as a heavily coupled quartet (2.65τ - 3.05τ). The isobutylenic protons appear as heavily coupled doublets at 4.72τ and 5.06τ presumably due to the deshielding effect of the benzene ring and long range coupling.

4a. Ethyl-p-tolyl ketone (125):

This ketone was prepared from toluene (18.4 gm), propionic acid (14.8 gm) by PPA condensation (PPA prepared from P_2O_5 (150 gm) and phosphoric acid (60 ml)) as described earlier. Yield 12 gm, b.p. $131^\circ\text{--}132^\circ\text{C}/25\text{--}27$ mm. The preparation was 95% pure by VPC.

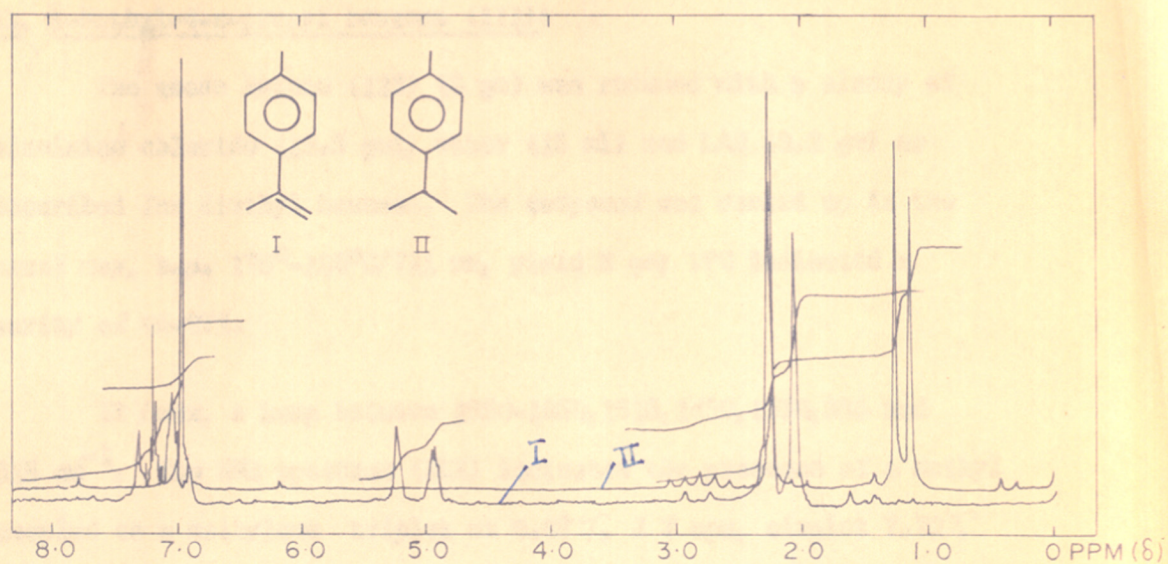


FIG. XL

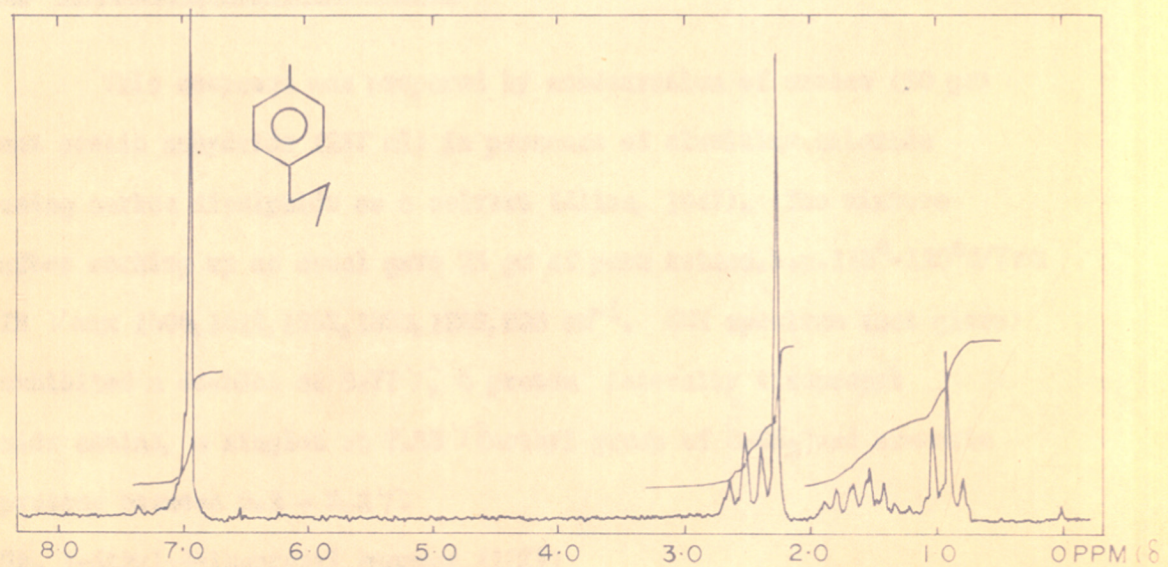


FIG. XLI

IR $\nu_{\max}$ 1680 (C=O), 1600, 1576, 832, 786 (p-substituted benzene), 950, 1345 (aromatic -C-CH₃), 1225 cm⁻¹.

4b. 1-Methyl-4-n-propyl benzene (111):

The above ketone (125) (5 gm) was reduced with a slurry of aluminium chloride (15.5 gm), ether (15 ml) and LAH (2.2 gm) as described for diethyl benzene. The compound was worked up in the usual way, b.p. 175°-180°C/711 mm, yield 3 gm; VPC indicated a purity of 96-97%.

IR $\nu_{\max}$, a hump between 1550-1650, 1515, 1450, 1370, 836 and 815 cm⁻¹. The NMR spectrum (XLI) indicated the presence of a methyl coupled to a methylene triplet at 9.07 τ , J 7 cps, singlet 7.72 τ , aromatic -C-CH₃, a singlet 3.05 τ (4 aromatic protons), a triplet (7.49 τ , J 8 cps) due to methylene group adjacent to the aromatic ring and complex series of signals (8.1 τ - 8.7 τ) ascribed to the remaining methylene group.

5a. Methyl-p-cumyl ketone (109):

This compound was prepared by condensation of cumene (30 gm) and acetic anhydride (237 ml) in presence of aluminium chloride using carbon disulphide as a solvent (Allen, 1947). The mixture after working up as usual gave 25 gm of pure ketone, b.p. 115°-120°C/7 mm. IR $\nu_{\max}$ 1696, 1615, 1563, 1358, 1265, 828 cm⁻¹. NMR spectrum (not given) exhibited a doublet at 8.77 τ , 6 proton intensity (isopropyl side chain), a singlet at 7.55 τ (methyl group of ¹C-CH₃) and aromatic protons between 2.2 - 3.2 τ .

5b. 1-Ethyl-4-isopropyl benzene (107):

The above ketone (10 gm) was reduced with a slurry of aluminium

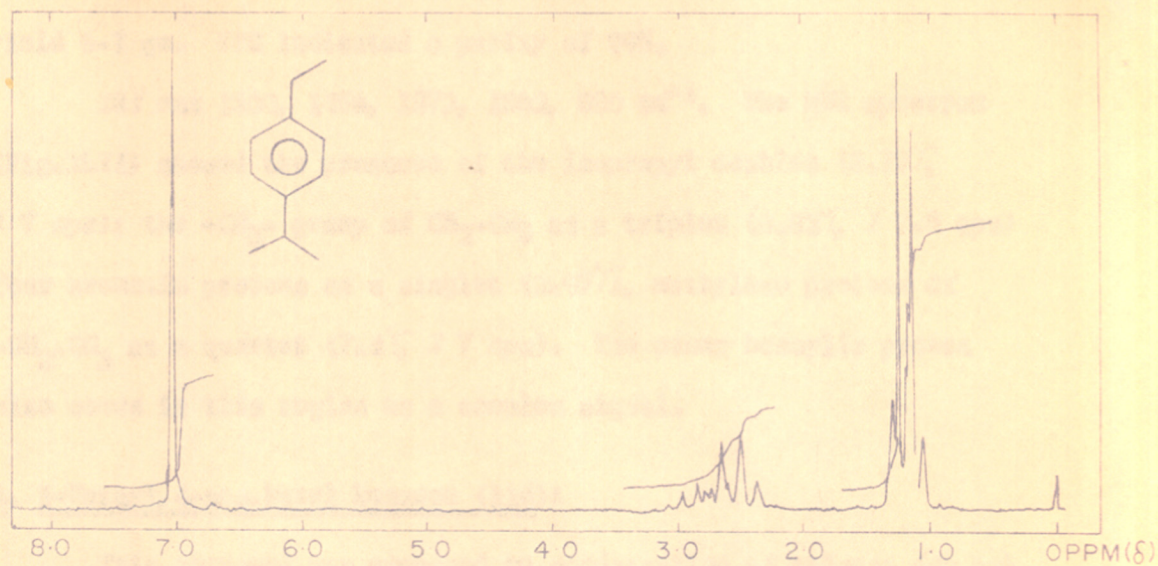


FIG. XLII

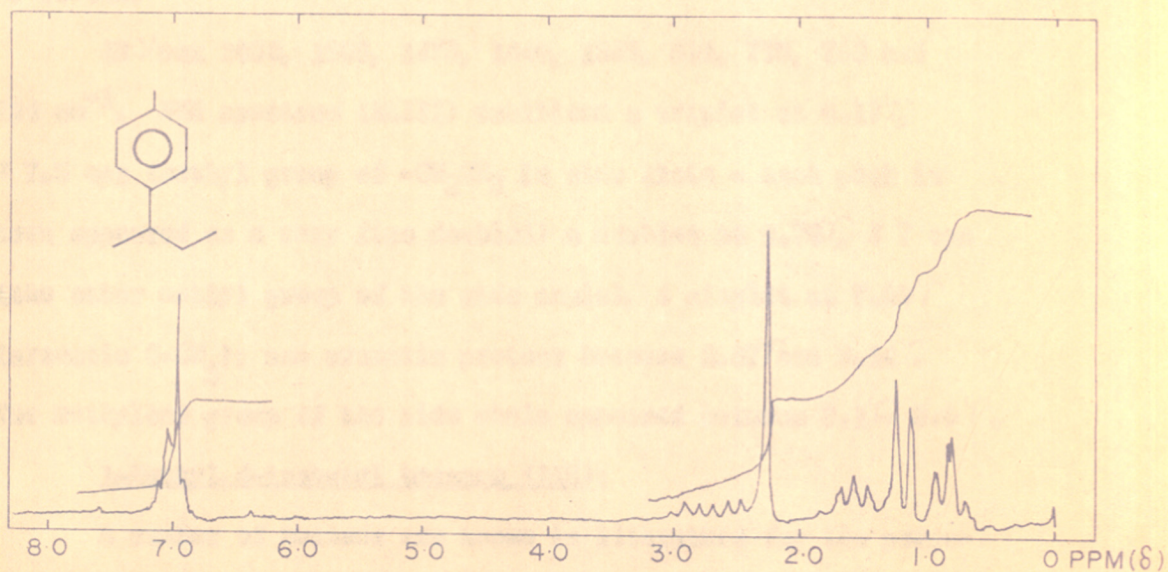


FIG. XLIII

chloride (30 gm) ether (40 ml) and LAH (4.5 gm) as described earlier. The reaction mixture was worked up as usual and the compound was purified by distillation. b.p. 186° - 195° C/709 mm or 65° - 70° /2 mm, yield 6-7 gm. VPC indicated a purity of 99%.

IR ν max 1503, 1456, 1372, 1362, 830 cm^{-1} . The NMR spectrum (Fig. XLII) showed the presence of the isopropyl doublet (8.77 τ , J 7 cps); the $-\text{CH}_2-$ group of CH_2-CH_3 as a triplet (8.82 τ , J 7.5 cps) four aromatic protons as a singlet (2.97 τ), methylene protons of $-\text{CH}_2-\text{CH}_3$ as a quartet (7.4 τ , J 7 cps). The other benzylic proton also comes in this region as a complex signal.

6. 1-Methyl,4-sec.butyl benzene (114):

This compound was prepared by condensation of toluene (46 gm) secondary butyl alcohol (37 gm) with five moles of 80% sulphuric acid at 70° as described by Mayer and Bernhauer (1929). The compound was worked up as usual and purified by distillation, b.p. 195° - 196° C/716 mm; yield 36 gm. The VPC indicated a purity of 98-99%.

IR ν max 2980, 1503, 1473, 1449, 1363, 813, 778, 758 and 724 cm^{-1} . NMR spectrum (XLIII) exhibited a triplet at 9.17 τ , J 7.5 cps (methyl group of $-\text{CH}_2\text{CH}_3$ in side chain - each peak in turn appeared as a very fine doublet) a doublet at 9.79 τ , J 7 cps (the other methyl group of the side chain), a singlet at 7.69 τ (aromatic $\text{C}-\text{CH}_3$); and aromatic protons between 2.87 τ and 3.14 τ . The methylene group of the side chain appeared between 8.1 τ -8.6 τ .

1-Methyl,4-isobutyl benzene (115):

A number of methods are known in literature for the preparation of this compound (Pajeau 1949, Pines et al, 1950, Urry and Nicolaides 1952, Somerville and Speerri 1952). In the present

studies it was prepared by the following method.

7a. Isopropyl-p-tolyl ketone (126):

This compound was obtained by condensation of toluene (10g) and isobutyric acid (9 g) with PPA in the manner described earlier. Yield 6 gm; VPC indicated a purity of 98-99%.

IR ν max at 1690 (C=O), 1618, 1565, 832 cm^{-1} (p-disubstituted benzene). The NMR spectrum (Fig. XLIV) supported the assigned structure (isopropyl group as a doublet 8.8%, J 7 cps; the aromatic C-CH₃ as a singlet at 7.64%; the aromatic protons as two characteristic doublets (2 protons each) at 2.89%, J 8 cps and 2.25%, J 8 cps. The lower group is due to down field shift as a result of conjugation with the carbonyl. The single proton in the isopropyl group is also shifted due to vicinity of the carbonyl and appears as a complex signal around 6.57%, J 7.5 cps.

7b. 1-Methyl, 4-isobutyl benzene (115):

This compound was obtained by reduction of the above ketone (126) (5 gm) with a slurry of aluminium chloride (15.4 gm), ether (15 ml) and LAH (2.2 gm) as described earlier. The reaction mixture after working up and distillation gave a compound b.p. 185°-188°C/711 mm, yield 3.3 gm. VPC indicated a purity of 98-99%. The IR ν max at 1510, 1455, 1370, 1362, 832 cm^{-1} (p-disubstituted benzene). The NMR spectrum (Fig. XLV) showed the isopropyl group (as a doublet at 9.15%, J 6.5 cps); aromatic C-CH₃ at 7.74%; four aromatic protons as a singlet at 3.09% and the methylene next to the ring as a doublet 7.6%, J 6.5 cps.

8. 1-Methyl, 4-tertiary butyl benzene (116):

This compound was prepared by condensation of toluene (100 gm)

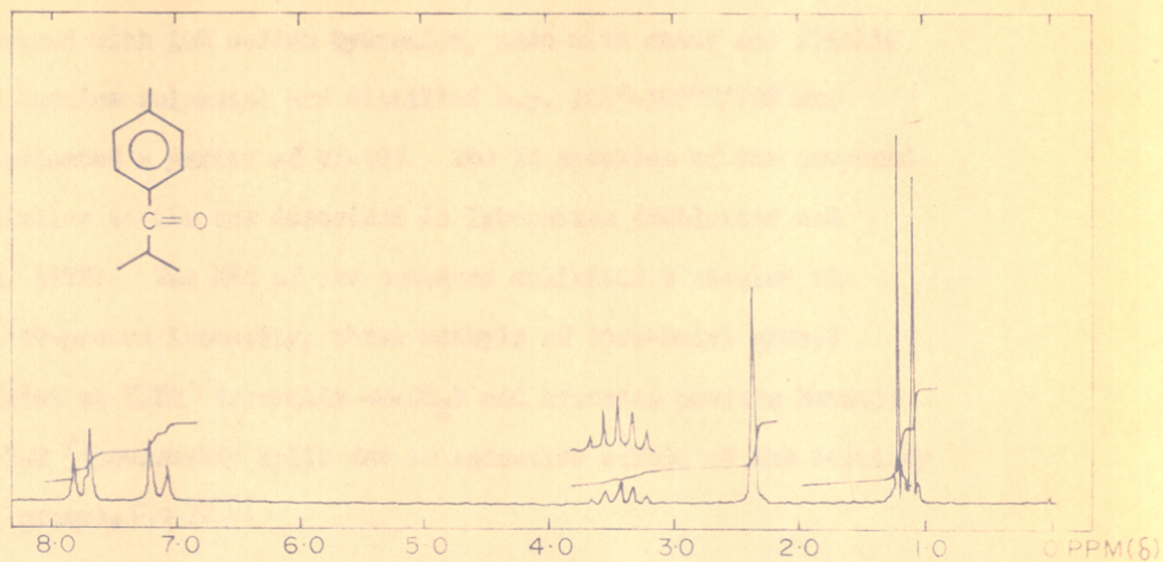


FIG. XLIV

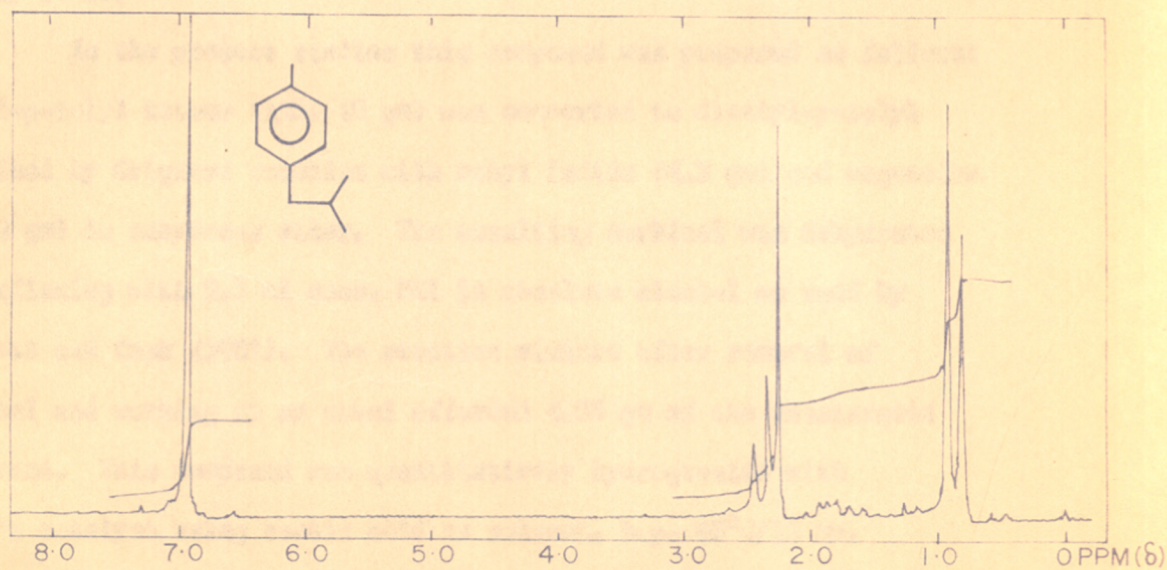


FIG. XLV

and isobutyl alcohol (25 ml) in presence of 80% sulphuric acid (Mayer and Bernhauer, 1929). The mixture was heated at the steam bath for 2½ hr with stirring. The hydrocarbon layer was separated and washed with 10% sodium hydroxide, then with water and finally dried (sodium sulphate) and distilled b.p. 183°-186°C/707 mm. VPC indicated a purity of 97-98%. The IR spectrum of the compound was similar to the one described in literature (Schlatter and Clark, 1953). The NMR of the compound exhibited a singlet at 8.72 τ (9-proton intensity, three methyls of tert-butyl group) a singlet at 7.72 τ (aromatic -C-CH₃) and aromatic protons between 2.74-3.2 τ (presumably split due to inductive effect of the tertiary butyl group). FIG XLVI

9. 1-Methyl-4-(1' ethyl propyl) benzene (117):

The method for the preparation of this compound as described in literature involves the condensation of 3 chloro pentane and toluene in presence of boron trifluoride as catalyst (Hennion and Kurtz, 1943).

In the present studies this compound was prepared as follows: Ethyl-p-tolyl ketone (125) (5 gm) was converted to diethyl-p-tolyl carbinol by Grignard reaction with ethyl iodide (4.3 gm) and magnesium (0.82 gm) in anhydrous ether. The resulting carbinol was dehydrated by refluxing with 2.5 ml conc. HCl in absolute alcohol as used by Barnett and Cook (1933). The reaction mixture after removal of alcohol and working up as usual afforded 4.53 gm of the unsaturated compound. This compound was quantitatively hydrogenated with Adam's catalyst using acetic acid as solvent. b.p. 95°C/13 mm. VPC indicated a purity of 93-96%. IR ν max 3000, 1515, 1460, 1370, 816 cm⁻¹.

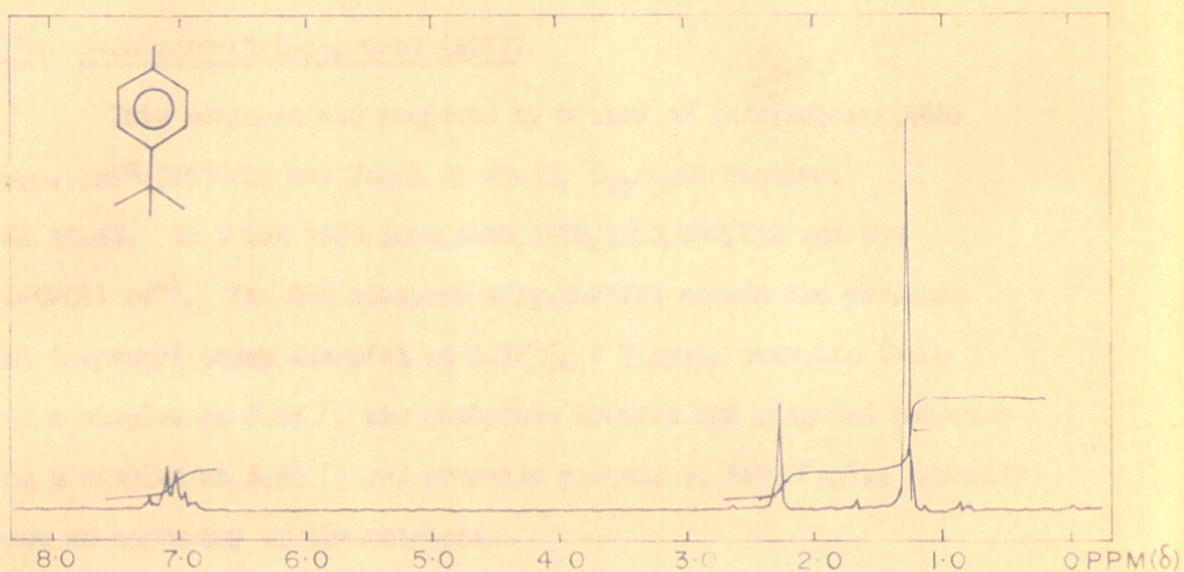


FIG. XLVI

NMR spectrum (Fig. XLVII) indicated a triplet of about 6 proton intensity at 9.27τ , J 6.5 cps (two methyls split due to methylenes), a singlet at 7.7τ (aromatic $C-CH_3$), four aromatic protons at 3.07τ and remaining methylene protons between 8.25τ and 8.75τ .

2-Methyl-p-cymene (120):

This compound was prepared by Grignard reaction of 2-chloromethyl-p-cymene (127) and decomposition of the complex with water (Compt. Rend. 186, 372).

10a. 2-Chloromethyl-p-cymene (127):

This compound was prepared by method of Turkiewicz (1940) b.p. 123° - 130° C/22 mm: Found Cl 20.1%, $C_{11}H_{15}Cl$ requires Cl 19.8%. IR ν max 1500, 1460, 1360, 1375, 1260, 830, 742 and 690 ($-C-Cl$) cm^{-1} . The NMR spectrum (Fig. XLVIII) showed the presence of isopropyl group (doublet at 8.77τ , J 7 cps), aromatic $C-CH_3$ as a singlet at 7.67τ , the methylene between the ring and chlorine as a singlet at 5.55τ ; and aromatic protons at 3.04τ split probably due to asymmetry of the molecule.

10b. 2-Methyl-p-cymene (120):

This compound was obtained by Grignard reaction of 2-chloromethyl-p-cymene (127) according to the procedure described by Vogel (1956) for benzyl chloride. The complex was decomposed with water and worked up as usual. b.p. 76° - 78° C/7-8 mm. VPC indicated a purity of 99%. IR ν max at 1615, 1570, 1510, 1472, 1362, 1378, 830 and 725 cm^{-1} . The NMR spectrum (Fig. XLIX) indicated the presence of isopropyl group (doublet at 8.78τ , J 7 cps) and the splitting

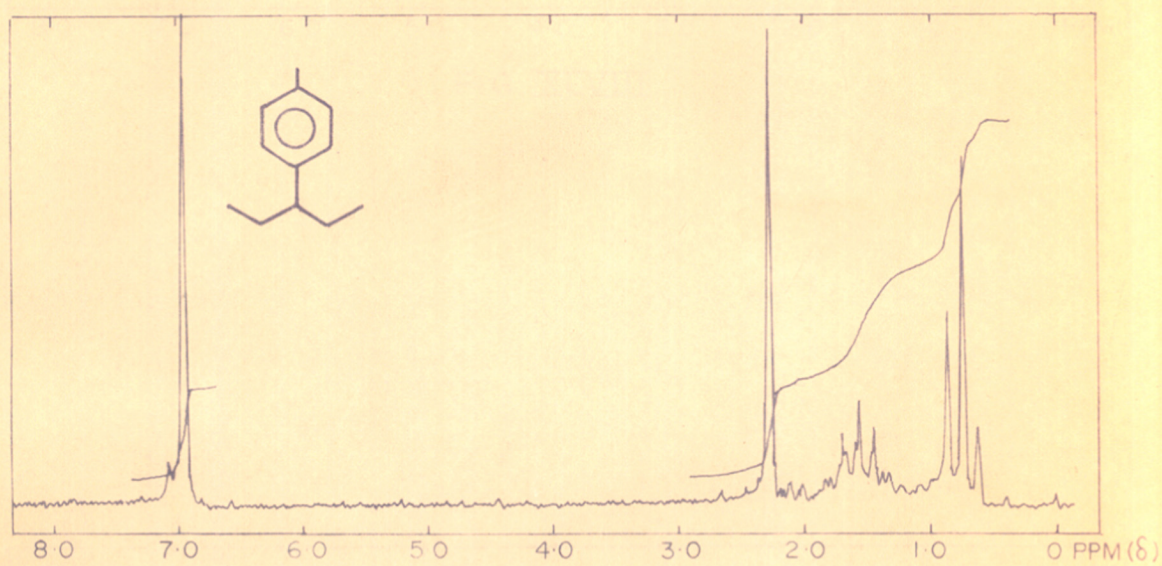


FIG. XLVII

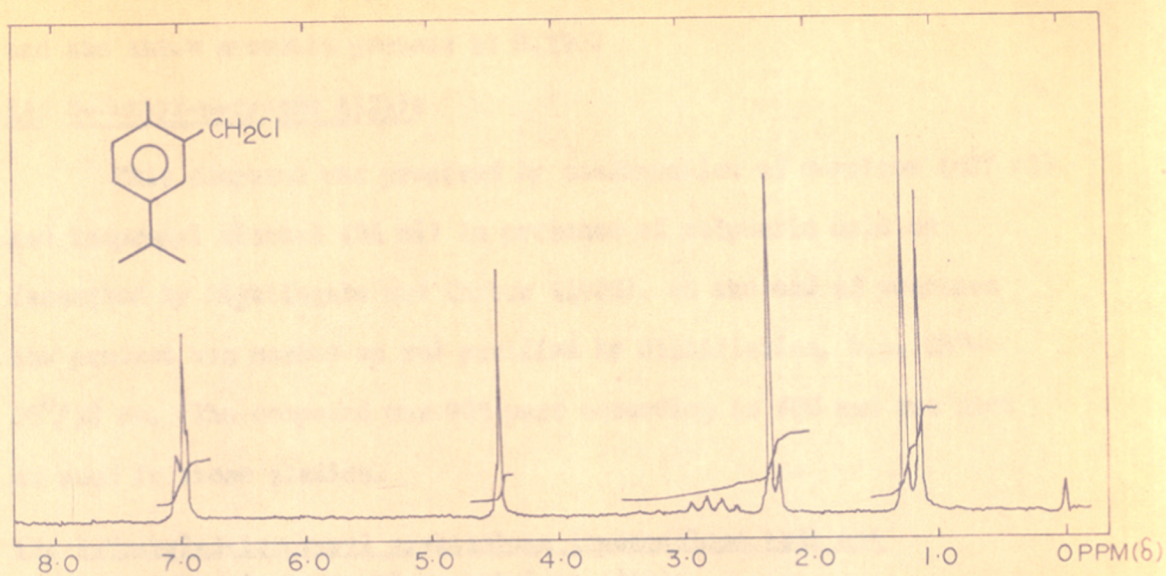


FIG. XLVIII

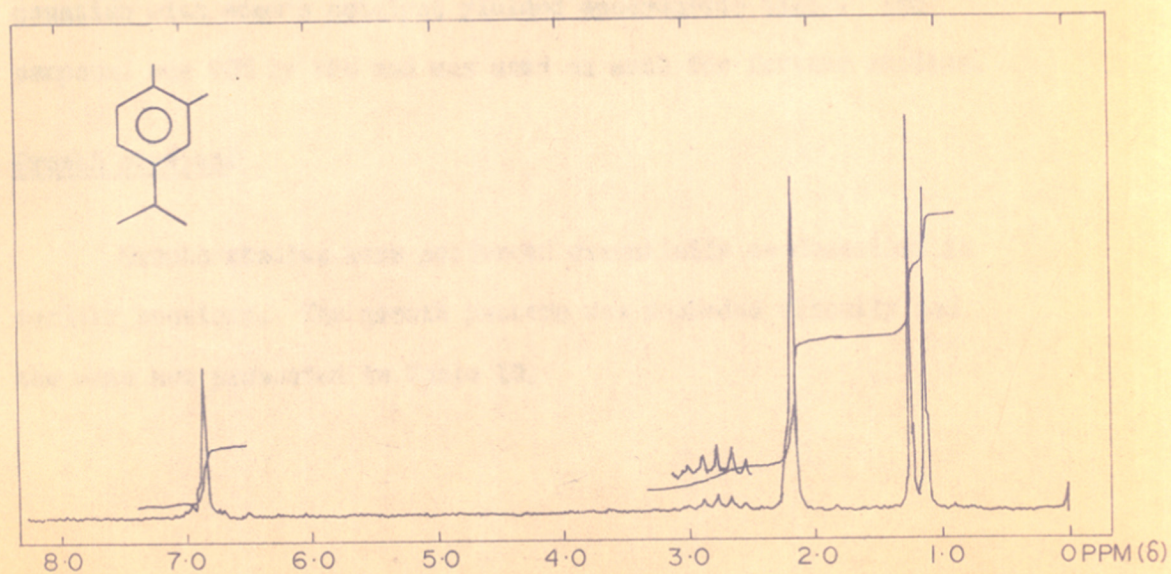


FIG. XLIX

proton between 7.2-7.4 τ , two aromatic $-\text{C}-\text{CH}_3$ groups at 7.8 τ and the three aromatic protons at 3.19 τ .

11. 3-Methyl-p-cymene (121):

This compound was prepared by condensation of m-xylene (487 ml) and isopropyl alcohol (24 ml) in presence of sulphuric acid as described by Nightingale and Carton (1940). At the end of reaction the product was worked up and purified by distillation, b.p. 83°-86°/13 mm. The compound was 90% pure according to VPC and was used as such in these studies.

12. 1-Methyl-4-isopropyl naphthalene (apocadalone, 118) and 1-methyl-4-isopropenyl naphthalene (119):

These compounds were prepared essentially according to method of Barnett and Cook (1933). The first step involved the condensation of α -methylnaphthalene with acetyl chloride in presence of aluminium chloride as catalyst and nitrobenzene as the solvent (Dziewonski and Marusinska, 1938). The resulting ketone was converted to carbinol by Grignard reaction which on dehydration yielded 1-methyl,4-isopropenyl naphthalene (119). This unsaturated compound on hydrogenation with Adam's catalyst yielded apocadalone (118). The compound was 90% by VPC and was used as such for further studies.

Growth studies:

Growth studies were performed essentially as described in earlier sections. The growth pattern was recorded visually and the data are presented in Table 10.

TABLE 10 (contd)

Substrates	TIME									
	HOURS		D A Y S							
	12	24	2nd	3rd	4th	5th	6th	7th	8th	10th
16 1-Methyl, 4-sec-butyl benzene (114)	traces	++ ^o	++	+++	+++	+++	discarded			
17 1-Methyl-4-(1'ethylpropyl)benzene (117)	-	-	-	-	-	-	-	-	-	-
18 1-Methyl, 4-isobutyl benzene (115)	yellow coloration	yellow coloration	+	+	+	++	++	++	++	++
19 Methyl-p-cumyl ketone (109)	-	-	-	-	-	-	-	-	-	-
20 p-Isopropylanisole (108)	-	-	-	-	-	-	-	-	-	-
21 1-Methyl-4-tert-butyl benzene (116)	-	-	yellow tinge	++ ^o	++ ^o	++ ^o	++ ^o	++ ^o	++ ^o	++ ^o
22 2-Methyl-p-cymene (120)	-	-	-	-	-	-	-	-	-	-
23 3-Methyl-p-cymene (121)	-	-	-	-	-	-	-	-	-	-
24 1-Methyl-4-isopropenyl naphthalene (119)	-	-	-	-	-	-	-	-	-	-
25 Apocadulene (118)	-	-	-	-	-	-	-	-	-	-
Substrate blanks of 9, 11, 13, 14 and 16	-	-	-	-	-	-	-	-	-	-

Inoculum: 2 ml of inoculum R at 640 mμ = 300 to 350. Substrate: 0.3 ml usually substrate added at suitable intervals to replenish the stock. In case of volatile compounds addition made at 12 or 24 hours. Temperature: 29°±2°C. Growth: - no growth; + feeble growth (Turbidity R around 100); ++ moderate growth (about 200), +++ good growth (above 250 to 300); ++++ heavy growth (R beyond 350-400 or above). ++^o yellow coloration; ++^o colour of the medium becomes pinkish and finally black.

CHAPTER VII

SUMMARY AND CONCLUSIONS

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The present studies were undertaken in order to investigate the modes of degradation of α and β -pinene by bacteria. A *Pseudomonas* isolated from soil by enrichment culture technique was found to grow on α and β -pinene. Among the other substrates on which it was able to grow limonene, Δ 1-p-menthene and p-cymene were important.

After identification of the culture, large scale fermentations were undertaken in order to isolate and characterise the transformation products. The ether and butanol extracts of the fermented broth were separated into neutral and acidic components.

The neutral fraction from α -pinene fermentation on chromatography yielded substantial amounts of d(+) borneol (47) identified by its physico-chemical characteristics and comparative IR spectroscopy with an authentic sample. The fraction moving slower on column than borneol could be demonstrated to contain myrtenol (49) by VPC in addition to borneol and small amounts of another unidentified hydroxylated compound.

From the acidic fraction a dicarboxylic acid has been separated through its sparingly soluble cyclohexylamine salt. The structure of this acid has been established as β -isopropyl pimelic acid (53).

Besides this dicarboxylic acid, the mother liquors contained a mixture of polar and nonpolar acidic fractions which were separated by partitioning of the methyl esters between petroleum ether and 90% aqueous methanol. The nonpolar methyl esters contained a fatty acid methyl ester in addition to a number of other components which

could not be separated. However, the presence of myrtenic (53), phellandric (59) and perillic acid (39) methyl esters was demonstrated in this fraction by comparative VPC.

The polar methyl esters on chromatography and saponification yielded an acid identified as oleuropeic acid (50). Another hydroxy ester (60) was also obtained by careful chromatography in the fractions moving slightly faster than oleuropeic acid methyl ester. The continuous ether extract yielded a dihydroxyacid of a probable structure 42.

The fermentation of β -pinene gave (-)-borneol, the saturated dicarboxylic acid (53) and appreciable amounts of oleuropeic acid (50). Small amounts of another hydroxy acid (tentative structure, 65) could also be isolated by column chromatography of the methyl esters. In addition a fatty acid and a monocarboxylic acid fraction similar to that from α -pinene were also obtained.

Adaptive enzyme studies revealed that borneol, myrtenol or even oleuropeic acid was not oxidised by pinene-grown cells whereas the monocarboxylic acid fraction was oxidised very rapidly. The dicarboxylic acid (53) was oxidised at a very slow rate. Growth and adaptive enzyme studies indicated that α or β -pinene-grown cells are also capable of oxidising limonene and Δ^1 -p-menthene with or without added chloramphenicol indicating the constitutive nature of limonene and Δ^1 -p-menthene degrading enzymes in these cells. It was also observed that the probable intermediates in oxidation of limonene and Δ^1 -p-menthene (perillic alcohol, (43), perillic acid (39), phellandrol (75), phellandric acid (59)) were oxidised rapidly by pinene grown cells. Based on the above observations a hypothetical scheme for the degradation of pinenes

by parallel pathways has been presented.

The first step in this scheme is the cleavage of the cyclobutane ring by attack of a proton on the double bond. The carbonium ion (66) on one hand is assumed to rearrange to give borneol as a side product. On the other, it rearranges to another carbonium ion (68) which may be the probable precursor of all the p-menthenoid compounds obtained in these studies. This carbonium ion probably participates in an enzyme-bound form in an internal hydride shift to form the carbonium ion (72) which may pick up a hydroxyl anion from the medium to give 4-terpineol - the possible precursor of the 4-hydroxy acids (61) and (65). The carbonium ion (72) can also be reduced stereospecifically to Δ -1-p-menthene by a suitable hydride donor.

Further degradation of Δ -1-p-menthene or limonene (obtained by elimination in the carbonium ion, 68) involves the progressive oxidation of the 7-methyl group of these compounds to the corresponding carboxy acids (39) and (59). These carboxy acids probably undergo hydration at the 1-2 double bond to give the hydroxy acids (40) and (77) which on subsequent oxidation of 2-hydroxyl group yield the corresponding unstable β -keto acids (45) and (78). These β -keto acids on cleavage give rise to dicarboxylic acids (41) and (53).

The presence of an NAD-alcohol dehydrogenase (for dehydrogenation of perillic alcohol, phellendrol and oleuropeic alcohol) and aldehyde dehydrogenase (for perillic aldehyde, phellendral, oleuropeic aldehyde) have been demonstrated in the cell-free extracts. Attempts to demonstrate the presence of cyclobutane ring cleaving enzyme in the intact cells under nitrogen and in the cell-free extracts were not successful.

In an attempt to understand the basic structural features necessary in the substrate molecule before it can serve as sole source of carbon for this bacterium and to obtain further support for the postulated pathways in Chapter V, a number of compounds were tested as growth substrates. Among the compounds with intact cyclobutane ring pinane (82), apopinene (91) failed to support growth indicating the essentiality of the double bond (for proton attack) and the 7-methyl group.

The organism has been found to utilise limonene, Δ 1-p-menthene (74), Δ 1-7 p-menthene (86), etc. but not the fully saturated p-menthane (83), Δ 8-p-menthene (84), 1-methyl cyclohexene (94) or Δ 3-carene (24). α -Terpineol (69) was also found to be inactive as a carbon source. Esterification or etherification of the hydroxyl group in α -terpineol did not make the resulting compounds acceptable to the bacterium as growth substrates. The significance and stereochemical implications of these findings are discussed.

The growth on p-cymene (97) offered an unique opportunity to extend the information regarding the behaviour of this organism towards model compounds. A number of compounds has been tested as growth substrates. The organism was capable of adapting most rapidly to p-cymene (97). Next in order were 1-methyl-4-sec-butyl benzene (114) and 1-methyl,4-n-propyl benzene (111). The organism took longer time to adapt itself to 1-methyl-4-ethyl benzene (102) and 1-methyl-4-isopropenyl benzene (112). 1-Methyl-4-isobutyl benzene (115) and 1-methyl-4-tert butyl benzene (116) were very poor growth supporters. 1-Methyl-4-ethyl benzene (102) appears to possess the basic minimum requirement in a compound before it can be acceptable to this organism as a growth substrate. None of the other compounds tested was capable of supporting growth of the organism. The above data are discussed

in the light of the proposed pathways for the degradation of
pinenes, limonene and p-cymene.

1. The first part of the paper is devoted to a discussion of the

importance of the problem of the origin of life.

2. The second part of the paper is devoted to a discussion of the

importance of the problem of the origin of life.

3. The third part of the paper is devoted to a discussion of the

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4. The fourth part of the paper is devoted to a discussion of the

importance of the problem of the origin of life.

5. The fifth part of the paper is devoted to a discussion of the

importance of the problem of the origin of life.

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